Cold preservation injury in organ transplantation:

beneficial effects of dopamine and carbon monoxide releasing molecules

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Eva Riedl

Two roads diverged in a yellow wood

Sorry I could not travel both

Be one traveler

Long I stood

And looked down one as far as I could

Robert Frost

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Chapter 1:

General introduction and aims of the study

1.1 History

The history of renal transplantation with the use of a kidney from a human cadaver donor began in 1933 and many more were performed since then (1-4). Although most of these were technically successful, none of the recipients had really benefited from this new approach for the treatment of end-stage renal failure. It was with the transplantation of a kidney from a monozygotic twin in 1953, that a new phase in the evolution of renal transplantation started (5). To date, renal transplantation has become common practice and the best alternative amongst other modalities of renal replacement therapy (6). Apart from renal transplantation, transplantation of other solid organs, e.g. heart, lung, liver, pancreas, have found their way in clinical practice and in recent decades thousands of recipients have substantially benefited from this treatment.

1.2 The main problems of organ transplantation

1.2.1 Donor shortage

Organ transplantation is unique among other surgical procedures, in that it critically depends on the donation of an organ or a partial organ from another person. Though since 1988 the number of organ donors has increased each year with an average of approximately 1100 organs, the number of patients on the waiting list has increased much more dramatic, resulting in an average increase of 5000 patients each year. Worldwide, more than 147,000 patients are waiting for a life-saving organ transplant, but only about 40,000 organ transplants are performed each year (7).

The need to bridge the gap between the increasing demand for organ donation and the relative stable supply of available organs have forced the transplant community to reevaluate donor selection criteria. Over the last decade, the ideal criteria for organ donor selection have been abandoned. Hence, more and more organs that would have been considered unsuitable for transplantation in the past are currently being used routinely. This has resulted in a new class of organ donors termed the "marginal" donors. The so called "expanded" donor pool, i.e. donors fulfilling the "extended criteria", consists of older donors or donors who have diabetes mellitus, hypertension, and renal insufficiency (8, 9). Today, the average donor is over 50 year-old and the main cause of death is intracranial hemorrhage (10). The risk factors that are associated with poorer transplantation outcome using marginal donors include age, previous

diseases with a systemic influence on the vascular system (i.e. arterial hypertension, diabetes mellitus), cause of death (cardiovascular or cerebrovascular disease), and brain death (35, 36).

Another alternative source for organ donors is the non-heart beating donor (NHBD). Death in these donors is defined as an irreversible cessation of circulatory and respiratory function. Thus, organs procured from NHBDs undergo prolonged hypotension and warm ischemia time. Historically, the use of NHBDs has been restricted to kidney donation, but, although limited, NHBDs have also been used in liver transplantation (11). A comparison of 377 NHBD kidney transplants to 12156 heart beating donor kidney transplants between 1995 and 1998 showed that though the NHBD group had inferior initial function, the 1-year and 3-year graft survival rates (85% and 73%) were not different from those in the heart beating donor group. Organs from NHBD may therefore be appreciated increasing importance for the cadaveric donor pool in the future (12).

1.2.2 Graft rejection

Vascularized organs, when transplanted into an immunocompetent recipient, are prone to immunological damage due to a variety of effector mechanisms. Several types of transplant rejection can be distinguished based on their time course of appearance and histological manifestation (13-15).

Hyperacute and acute vascular rejection are both directed against donor endothelial cells, but they differ markedly in the onset, histology and mechanisms of destruction of the vasculature. Whereas hyperacute rejection immediately starts after reperfusion of the transplant, and is mediated by pre-existing antibodies and complement, acute vascular rejection starts within days to weeks and the relative importance of antibodies and complement in the pathogenesis of vascular rejection is unclear (16, 17). Acute vascular rejection is characterized by a profound thrombus formation and focal ischemia often associated with neutrophil and mononuclear cell infiltration (18). In contrast, in hyperacute rejection neutrophil infiltration is mostly lacking.

Acute interstitial rejection differs from the former two types, in that it is defined by the infiltration of mononuclear cells into the tubuli, also referred to as ‘tubulitis’ (19). In fact, tubular cells, as opposed to endothelial cells in hyperacute and acute vascular rejection, are the target cells that are destroyed in this type of rejection (20, 21). Acute interstitial rejection

occurs mostly within the first three months after transplantation and is exclusively mediated by alloreactive T-cells that recognize donor antigens on tubular cells.

Chronic rejection is the most prominent factor for graft loss after transplantation. In renal allografts, chronic rejection, also termed chronic allograft nephropathy, is characterized by a relative slow rate of decline in renal function. The histopathological findings in chronic allograft nephropathy are atherosclerosis, glomerular sclerosis, multilayering of the peritubular capillaries, interstitial fibrosis and tubular atrophy (22).

1.3 Relation between pre-transplantation injury and graft survival

1.3.1 Response to injury hypothesis

The hypothesis that tissue injury initiates allograft rejection was put forward in 1995 by Lu et al (23) and contains two implications. On one hand it postulates that tissue injury of the allograft is recognized by the immune system in the recipient. On the other hand it postulates that such recognition initiates allograft rejection. It should be noted however, that the presence of alloantigens is required to maintain immune reactivity. Several sets of observations support this hypothesis. Firstly, it has been demonstrated that in renal biopsies from autografts in dogs and allografts from identical twins, a mild inflammatory infiltration is still present (24). These grafts are not rejected since no mismatched alloantigens are present on the grafts, thus there is no alloimmune reaction in these cases. The observed inflammation must be due to injury during the transplant procedure. Secondly, if injury initiates rejection, severe injury should result in a greater incidence of rejection. Indeed, ample evidences suggest that severe injury makes renal allografts more prone to rejection (25). Thirdly, if injury initiates rejection, less injury should result in less rejection. This is also compatible with the observation of Terasaki et al (26) that transplant survival of renal allografts from living donors is superior to that of cadaver donors. Finally, if tissue injury initiates rejection, therapies which decrease injury during transplantation should also decrease the incidence of acute rejection. In fact, there are reports showing that the use of human recombinant superoxide dismutase and antibodies against intercellular adhesion molecule 1 (ICAM-1) or lymphocyte function associated antigen 1 (LFA-1), all of which reduce the extent of ischemia and reperfusion injury, can decrease acute rejection episodes (27-29).

1.3.2 Living donors versus cadaveric donors

Based on the kidney transplant data of the United Network of Organ Sharing (UNOS) renal transplant registry, Terasaki et al (26) reported that the three-year graft survival of kidneys from living donors was much higher than that of kidneys from cadaver donors, despite a better HLA matching of the latter. This indicates that poor survival of grafts from cadaver donors can not be solely attributed to the difference in immunogenicity. According to the response to injury theory, the higher survival rate of renal transplants from living donors compared to that of cadaver donors should also be attributed to the fact that the latter suffer from more damage. All renal allografts suffer unavoidable injury from the transplant process during organ retrieval and the actual transplantation. However, allografts from cadaver donors are further injured by hemodynamic instability associated with brain death, trauma or acute illness in the donors and by prolonged cold storage while in transit from the donor to the recipient (30-33).

The advantages for the use of a living donor in renal transplantation are summarized as follows: less pre-transplantation injury, better long-term patient and allograft survival, lower incidence of rejections, less immunosuppression, and reduced need for post-transplantation hospital visits and readmissions (34).

1.3.3 Pre-transplantation injury

1.3.3.1 Brain death

Brain death is a dynamic course of events that influences organ allograft quality. At the onset of brain death, massive quantities of catecholamines are released within seconds, which causes an increase in heart rate and leads to vasoconstriction with increased vascular resistance and blood pressure (37-39). The more acutely brain death is imposed, the higher the peak catecholamine levels are (39). Already 30 seconds after brain death serum concentration of nonadrenaline, adrenaline and dopamine are significantly increased, and fall below baseline levels within one hour after brain death (40). Ultimately, a state of hypoperfusion is reached. This “autonomic storm” is believed to have important consequences for organ perfusion, inflammation and thereby damage to peripheral organs (41-43).

Since brain death promotes inflammation in end-organs, affects hormone regulation and hemodynamic stability, it is generally accepted that it severely influences graft quality (44). Moreover, brain death is often associated with ischemia-reperfusion (I/R) injury (45) which

can further augment the proinflammatory state of the graft and thus increase graft immunogenicity (46, 47). In the sequel of brain death a rapid up-regulation of inflammatory mediators like IL-6 and TNFα occurs (48-51). This might in turn result in the upregulation of an array of genes including selectins, fibrinogen and kidney injury molecue 1 (KIM-1) (52). Brain death is considered to be a risk factor for organ dysfunction (43, 53) and accelerates acute rejection episodes (54-56).

1.3.3.2 Cold preservation

Allocation of donor organs has been made possible by success of organ preservation. The principles of organ preservation are hypothermia, prevention of cell swelling and acidosis (57). Hypothermia is an essential mean for slowing down cell metabolism and delaying injurious processes provoked by the deficiency of oxygen. Although hypothermic organ preservation is common practise in transplantation of solid organs, prolonged cold storage may lead to tissue damage and thereby it may have adverse effects on long-term transplantation outcome (58, 59).

There are at least four components that are involved in cold ischemic transplant injury: the coupled effect of ischemia and hypothermia during cold storage and the coupled effect of reperfusion and rewarming after transplantation (69). The known mechanisms of cold ischemic injury are related to perturbations in osmoregulation, energetics, and aerobic metabolism (60). During hypothermic preservation, lack of oxygen leads to a rapid decline of intracellular ATP concentrations (61). Consequently, energy depletion leads to dissipation of ion gradients across the cell membrane as a result of an impaired function of the Na-K ATPase. Eventually, intracellular calcium increase (64) which in turn leads to mitochondrial calcium overload (66). Ischemia *per se* stimulates glycogenolyse, most likely via release of inosine (87), resulting in production of lactate and initiation of cellular acidosis (61).

A number of studies have demonstrated that release of intracellular iron plays an important role in cell damage during cold preservation (65, 70). However, the intracellular source for this release has not clearly been defined. Since free iron leads to the generation of toxic hydroxyl radicals, and synthesis of glutathione and intracellular antioxidants are abolished as a result of ATP depletion, reactive oxygen species accumulate (62, 63), giving rise to massive cell destruction (66, 69).

Endothelial injury is a prominent feature of cold ischemia (67). In a rat model, it has been reported that renal allografts exposed to cold ischemia exceeding 6 hours develop progressive vascular injury and inflammation 24 hours after transplantation. This was characterized by an irregular PECAM staining and augmented VCAM-1 expression, accompanied by perivascular monocyte infiltration (71).

Other studies have also demonstrated that prolonged cold ischemia is associated with more inflammation (72). Recipients of grafts with long cold ischemia experience early acute rejection more often than those with minimal cold ischemia and are at higher risk for delayed graft function (68, 73, 74). Thus, prolonged cold preservation severely influences long-term graft survival (59, 68).

1.4 Donor preconditioning

Because donor factors are important determinants of short and long-term allograft survival (75), optimal management of cadaver donors is clearly of high relevance for transplantation outcome. In this respect, the value of donor preconditioning has been appreciated and many different approaches to achieve this objective were successful. Most, if not all, of these strategies were aimed at reducing ischemia reperfusion (I/R) injury, a complex interrelated sequence of events that classically involves the vascular endothelium and activated leukocytes (76-79).

It is important to emphasize that donor preconditioning has great advantages because it not only might improve transplantation outcome, but can also be achieved cost-efficiently without great effort or side effects for the recipient (96). Based on the principle being used, donor preconditioning can be divided into ischemic preconditioning and pharmacological preconditioning.

1.4.1 Ischemic preconditioning

In recent years, extensive studies have expanded the knowledge of the role of preconditioning for cardiac protection against IR injury. Exposing the heart to brief episodes of ischemia protects the myocardium and vascular endothelium against functional damage and cell death caused by subsequent prolonged ischemia, a phenomenon that is called ischemic preconditioning (IP) (80). The mechanisms of IP is complex and involves a variety of mediators and second messengers such as adenosine, protein kinase C, ATP-dependent

potassium channels and oxygen radicals (80-82). In addition to the heart, a beneficial effect of IP has been reported for liver, lung and intestinal allografts in experimental models (83-85). Both an early and late phase of protection has been identified in IP. In the early phase it is believed that IP preserves energy metabolism during sustained ischemia (86). Adenosine monophosphate activated protein kinase (AMPK) protects cells by acting as a low-fuel warning system that is switched on by ATP depletion (86). Whereas activation of AMPK concomitantly reduces ATP depletion, lactate accumulation and cell injury, inhibition hereof abrogates the beneficial effect of IP of liver allografts (86).

The L-arginine nitric oxide (NO) pathway also seems to play a pivotal role in the protective effect of IP in both the early and late phase. This is based on the observations that preconditioning with inhaled NO protects against IR injury (83) and treatment of animals with nitric oxide synthase inhibitor diminishes the effect of IP (85).

1.4.2 Pharmacological preconditioning

Pharmacological preconditioning can be accomplished by treating the donor or allograft with compounds that can either prevent organ damage due to ischemia or prevent cell activation or infiltration of mononuclear cells upon reperfusion.

As transendothelial migration of inflammatory cells relies on the presence of adhesion molecules on the endothelium, such as ICAM, VCAM, selectins, and their ligands on leukocytes (76-79), prevention of this particular interaction or expression of adhesion molecules is a widely used approach in animal models to limit infiltration of mononuclear cells into the allograft. The use of anti-sense ICAM constructs (88), and soluble ligands or antibodies against adhesion molecules have been shown to be successful in IR models (89-91).

The transcriptional activation of inflammatory genes is tightly regulated by transcription factors, including nuclear factor–κB (NF–κB) (92-94). By perfusion a renal allogaft with NF-κB decoy oligodeoxynucleotides prior to transplantation, a significant decrease in monocyte infiltration was observed (95). In addition, other studies have shown that donor pre-treatment with steroids significantly decreases tissue and serum expression of proinflammatory cytokines in brain dead human donors (97). Recently, carbamylated recombinant human erythropoietin (CEPO) was also demonstrated to be neuroprotective and renoprotective in experimental models of brain injury (98), cerebral ischemia (99) and brain death (100).

Catecholamines are frequently used in donor management to improve blood pressure. In two independent studies, Schnuelle et al found that donor catecholamine usage reduced the incidence of acute rejection episodes within the first month after transplantation and improved long term allograft survival after kidney transplantation of cadaveric allografts (103, 104). In rat models, dopamine pre-treatment was found to inhibit tubulitis (105) and to improve both short and long term outcome (106) after renal transplantation. Several possible mechanisms may account for this phenomenon. Firstly, catecholamines have the propensity to protect endothelial cells against cold preservation injury (107). Cold preservation also impairs endothelial barrier function, which is restored during rewarming when endothelial cells were pre-treated with catecholamines prior to cold storage (108). Therefore, dopamine pre-treatment can maintain vascular integrity by limiting vascular permeability of solid organs. Secondly, catecholamines may mitigate the detrimental effect of acute traumatic brain death on the donors. As acute brain death is associated with a profound neuroendocrine dysregulation, often leading to hypotension, application of catecholamines may improve hemodynamic stability in these donors. Consistent with this is the observation by Schnuelle et al that the salutary effect of catecholamines was most pronounced in donors that had suffered acute traumatic brain death (103). Thirdly, explosive brain death is associated with cytokine production, expression of adhesion molecules and inflammation (41, 97). While catecholamines have been shown to be able to reduce the expression of these inflammatory mediators, this might contribute to the protective effect of donor catecholamine pre-treatment (102, 109). Catecholamines can also induce the production of the anti-inflammatory cytokine IL-10 in monocytes (110, 111). Fourthly, dopamine is capable to stimulate the induction of protective enzymes HO-1 in renal tubular epithelial and endothelial cells (112, 113). The induction of HO-1 has been reported to exert beneficial effects in a number of transplantation models (101), rendering the organ more resistant to the insult of ischemia reperfusion and inflammation.

Recently, the use of gaseous agents for donor pre-treatment has gained more interest. Carbon monoxide (CO), a byproduct of heme catalysis by heme oxygenase, has shown to be cytoprotective as it induces vasorelaxation (114, 115), inhibits cell proliferation (116, 117) and apoptosis (118), suppresses inflammation (119, 121), protects organs against I/R injury (120, 122), and diminishes graft rejection (123). Low dose CO treatment also ameliorates ischemia/reperfusion injury of transplanted lung, liver, intestines and kidney in rat models (120, 124-126). As a novel approach to deliver CO, Motterlini et al (127, 128) has recently

developed a class of compounds, termed CO releasing molecules (CORMs), which are able to release CO in a controllable manner (129). Like CO, CORMs have potent anti-inflammatory effects (119), and improve vascular function by exerting significant vasodilation in rat aortas precontracted with phenylephrine (130).

Nitric oxide (NO), normally synthesized from L-arginine by NO synthase, shares at least one common mechanism of action with CO, i.e. they both stimulate guanylate cyclase. The beneficial effect of NO has been demonstrated in mouse models using eNOS deficient donor (131). Cold ischemia/reperfusion injury in liver grafts from wild type donors was significantly attenuated compared to eNOS-deficient donors after liver transplantation (131). Although eNOS is an important source of NO production, during ischemia the endogenous nitrite pool may serve as alternative NO source when eNOS activity is compromised as a result of decreased oxygenation and increased acidosis (132). Recently, data from a number of experiments showed that nitrite administration before transplantation protects heart, liver and kidney against ischemia/reperfusion injury (133).

Hydrogen sulphide (H_2S) is a third gaseous mediator that protects against ischemia/reperfusion injury (134). Although these data look promising, more work is required for the potential use of H_2S as a cytoprotective mediator in organ transplantation.

1.5 Aims of this study

Graft immunogenecity is not completely dependent on HLA differences between donor and recipient. Although allocation of renal allografts is based on HLA matching, the importance of this is often ignored due to the small (10%) difference in graft survival rates between the best and worst matched pairs (135). Also in liver transplantation, neither matching for HLA class I nor HLA class II has shown to influence transplantation outcome (136). As kidney transplants from unrelated living donors are performing exceedingly well despite poor HLA compatibility, the concept of tissue injury as important determining factor for long-term allograft survival has been more appreciated. Attempts should therefore be undertaken to limit tissue injury of the allograft before organ implantation. Cold preservation is a major cause of pre-transplantation injury, and hence it represents an important target for intervention. Since in retrospective studies the beneficial effect of donor catecholamine usage has been demonstrated, the aim of this thesis was to further elucidate in *in vitro* and *in vivo* models why catecholamines are protective. Emphasis was put on the vasculature because prolonged hypothermia is associated with endothelial barrier dysfunction, the endothelium is a major

player in inflammation, and above all, the vasculature is severely affected in chronic allograft vasculopathy. In particular, we assessed in an isolated ventilated and perfused lung model how cold preservation affects oedema formation and inflammation and if this was diminished by dopamine treatment. Furthermore, we made use of *in vitro* models to assess the processes that might lead to preservation injury and how these were influenced by dopamine. Efforts were also made to identify the structural entities within catecholamines that convey protection. Apart from catecholamines we also investigated CO-releasing molecules (CORM) as a mean to limit inflammation and to improve vascular integrity during cold preservation.

References

1. Hume DM, Merril JP, Miller BF, Thorn GW. Experience with renal homotransplantation in human; report of nine cases. *J Clin Invest* 34:327,1995
2. Krieg AF, Bolande RP, Holden WD, Hubay CA, Persky L. Membranous glomerulonephritis in a human renal homograft. Am J Clin Path 34:155, 1960
3. Michon L, Hamburger J, Oeconomos N et al. Une tentative de transplantation renale chez I'homme: aspects medicaux et biologiques. Press Med 61:1419,1953
4. Murray G, Holden R. Transplantation of kidneys, experimentally and in human cases. Am J Surg 87:508, 1954
5. Murray JE, Merril JP, Harrison JH. Renal homotransplantation in identical twins. Surg Forum 6:432,1955
6. Schnueller P, Lorenz D, Trede M, van der Woude FJ. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence fro reduced mortality risk compared with hemodialysis during long-term follow-up. J Am Soc Nephrol 9:2135-2141,1998
7. Josep Lloveras, the 16th International Congress of the Transplantation Society, Barcelona
8. Fijter JW, Mallat MJK, Doxiadis IIN, et al. Increased immunogenicity and cause of graft loss of old donor kidneys. J Am Soc Nephrol. 12:1538-1546, 2001
9. Kasiske BL, Snyder J. Matching older kidneys with older patients does not improve allograft survival. J Am Soc Nephrol. 13: 1067-1072, 2002
10. Bos EM, Leuvenink HGD, van Goor H, Ploeg RJ. Kidney grafts from brain dead donors: inferior quality or opportunity for improvement? Kidney Int. 72: 797-805, 2007
11. Malaise J, Van Deynse D, Dumont V et al. Non-heart-beating donor, 10-year experience in a Belgian transplant center. Transplant Proc 2007; 39: 2578-9
12. Land W. Organ shortage: the problem and potential solutions: transplantation of marginal organs outcome analysis-kidney. Program and abstracts from the first joint annual meeting of the American society of transplant surgeons and the American society of transplantation; May 13-17, 2000; Chicago
13. Morris RE. Vasular and cellular mechanisms of chronic renal allograft dysfunction. Transplantation 71 (S11):SS37,2001
14. Yilmaz S, Nutley M, Taskinen E, Paavonen T, Hayry P. Post-transplantation histology as surrogate marker for long-term kidney allograft outcome. Ann Transplant 5:37,2000
15. Baltzan M, George D. Renal allograft pathology: The Banff classification. Kidney Int 56:1603,1999
16. Platt JL, Saadi S. The role of complement in transplantation. Mol Immunol 36:965,1999
17. Davis EA, Pruit SK, Greene PS, Ibrahim S, Lam TT, Levin JL. Inhibition of complement evoked antibody, and cellular responses prevents rejection of pig to primate cardiac xenografts. Transplantation 62:1018,1996
18. Platt JL, Fischel RH, Matas AJ, Reif SA, Bolman RM, Bach FH. Immunopathology of hyperacute rejection in a swine-to-primate model. Transplantation 52:214,1991
19. Moolenaar W, Bruijn JA, Schrama E et al. T-cell receptors and ICAM-1 expression in renal allografts during rejection. Transpl Int 4:140,1991
20. Yard BA, Kooymans-Couthino M, Reterink T et al. Analysis of T-cell lines from rejecting renal allografts. Kidney Int Suppl 39:S133,1993
21. Yard BA, Claas FH, Paape ME et al. Recognition of a tissue-specific polymorphism by graft infiltrating T-cell clones isolated from a renal allograft with acute rejection. Nephrol Dial Transplant 9:805, 1994
22. Paul LC. Chronic allograft nephropathy: an update. Kidney Int 56:783,1999
23. Lu CY, Penfield JG, kielar ML, Vazquez MA, Jeyarajah DR. Hypothesis: is renal allograft rejection initiated by the response to injury sustained during the transplant process? Kidney Int 55:2157-2168, 1999
24. Porter KA. Renal transplantation, in pathology of the kidney edited by Heptinstall RH, Boston Little, Brown & Co., 1799-1934, 1992
25. Halloran P, Aprile M, Farewell V, Ontario Renal Transplantation Research Group. Factors influencing early renal function in cadaveric kidney transplants: A case-control study. Transplantation 45:122-127, 1987
26. Terasaki PI, Cecka JM, Gjetson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unreleated donors. N Eng J Med 333:333-336, 1995
27. Land W, Schneeberger H, Schleibner ST et al. Beneficial effect of human recombinant superoxide dismutase on both acute and chronic rejection events in recipients of cadaveric renal transplants. Transplantation 57:211-217, 1994
28. Kelly KJ, Williams WWJ, Colvin RB, Bonventre JV. Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. Proc Nat Acad Sci 91:812-816, 1995
29. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and IFA-1. Science 255:1125-1127, 1992

30. Goujon JM, Vandewalle A, Baumert H, Carretier M, Hauet T. Influence of cold storage conditions on renal function of autotransplanted large pig kidneys. Kidney Int 58:838-850, 2000
31. Lazar N, Dallos G, Nemes B, Nemeth T, Sotonyi P, Kobori L. Experimental investigation of preservation injury in animal kidneys after reperfusion with Euro-Collins. Acta Chir Hung 36:192-194, 1997
32. Van der hoeven JA, Lindell S, Schilfgaarde R et al. Donor brain death reduces survival after transplantation in rat livers preserved for 20hr. Transplantation 72:1632-1636, 2001
33. Wilhelm MJ, Pratschke J, Beato F et al. Activation of the heart by donor death accelerates acute rejection after transplantation. Circulation 102:2426-2433, 2000
34. Curtis J. Organ shortage: the problem and potential solutions: risk benefits of living donation kidney. Program and abstracts from the first joint annual meeting of the American society of transplant surgeons and the American society of transplantation; May 13-17, Chicago, 2000
35. Tullius SG, Volk H-D, Neuhas P. Transplantation of organs from marginal donors. Transplantation. 72:1341-1349, 2001
36. Port FK, Bragg-Gresham JL, Metzger RA et al. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. Transplantation. 74:1281-1286, 2002
37. Novitzky D. Selection and management of cardiac allograft donors. Curr Opin Cardiol. 11:174-182, 1996
38. Novitzky D, Wicomb WN, Cooper DK, Rose AG, Reichart B. Prevention of myocardial injury during brain death by total cardiac sympathectomy in the Chacma baboon. Ann Thorac Surg 41:520-524, 1986
39. Shivalkar B, van Loon J, Wieland W et al. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. Circulation 87:230-239, 1993
40. Herijgers P, Leunens V, Tjandra-Mage TB, Mubagwa K, Flameng W. Changes in organ perfusion after brain death in the rat and ist relation to circulating catecholamines. Transplantation 62:330-335, 1996
41. Marschall VC. Pathophysiology of brain death: effects on allograft function. Transplant Proc 33:845-846, 2001
42. Kusaka M, Pratschke J, Wilhelm MJ et al. Activation of inflammatory mediators in rat renal isografts by donor brain death. Transplantation 69:405-410, 2000
43. Van der Hoeven JA, Ploeg RJ, Postema F et al. Induction of organ dysfunction and up-regulation of inflammatory markers inthe liver and kidney of hypotensive brain dead rats: a model to stuga marginal organ donors. Transplantation 68:1884-1890, 1999
44. Pratschke J, Neuhaus P, Tullius SG. What can be learned from brain-death models? Transplant Int 18: 15-21, 2005
45. Goes N, Nrmson J, Ramassar V, Halloran PF. Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor-beta 1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. Transplantation 65: 1533-42, 1998
46. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 12: 991-1045, 1994
47. Van der Woude FJ. Graft immunogenicity revisited: relevance of tissue-specific immunity, brain death and donor pre-treatment. Nephron 91: 181-7, 2002
48. Huber TS, Kluger MJ, Harris SP, D'Alecy LG. Plasma profiles of IL-6-like and TNF-like activities in brain-dead dogs. Am J Physiol. 261(5 Pt 2):R1133-40, 1991
49. Skrabal CA, Thompson LO, Potapov EV et al. Organ-specific regulation of pro-inflammatory molecules in heart, lung, and kidney following brain death. J Surg Res. 123(1):118-25, 2005
50. Takada M, Nadeau KC, Hancock WW et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation 65: 1533-42, 1998
51. Schuurs TA, Morariu AM, Ottens PJ et al. Time-dependent changes in donor brain death related processes. Am J Transplant. 6(12):2903-11, 2006
52. Schuurs TA, Gerbens F, van der Hoeven JA et al. Distinct transcriptional changes in donor kidneys upon brain death induction in rats: insights in the processes of brain death. Am J Transplant. 4(12):1972-81, 2004
53. Van der Hoeven JA, Molema G, ter Horst GJ et al. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. Kidney Int. 64(5):1874-82, 2003
54. Praschke J, Wilhelm MJ, Kusaka M, Basker M, Cooper DK, Hancock WW, Tilney NL. Brain death and its influence on donor organ quality and outcome after transplantation. Transplantation. 67(3):343-8, 1999
55. Praschke J, Wilhelm MJ, Kusaka M et al. Accelerated rejection of renal allografts from brain-dead donors. Ann Surg. 232(2):263-71, 2000
56. Praschke J, Wilhelm MJ, Kusaka M, Hancock WW, Tilney NL. Acute rejection of rat renal allografts is accelerated by donor brain death. Transplant Proc. 31(1-2):874-5, 1999
57. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet 2: 536-538, 1967
58. Salahudeen AK. Consequences of cold ischemic injury of kidneys in clinical transplantation. J Investig Med. 52:296-8, 2004

59. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int 65:713-718, 2004
60. Belzer FO, Southard JH. Principles of solid organ preservation by cold storage. Transplantation 45:673-676, 1988
61. Lang F, Busch GL, Gulbins E. Physiology of cell survival and cell death: implications for organ conservation. Nephrol Dial Transplant 10:1551-1555, 1995
62. Meister A. Glutathione metabolism and its selective modification. J Biol Chem. 263: 17205-17208, 1988
63. Slusser SO, Grotyohann LW, Martin LF, Scaduto RC. Glutathione catabolism by the ischemic rat kidney. Am J Physiol. 258: F1547-1553, 1990
64. Lang F, Messner G, Wang W, Oberleithner H. Interaction of intracellular electrolytes and tubular transport. Klin Wochenschr. 61:1029-1037, 1985
65. Rauen U, Kerkweg U, Wusteman MC, de Groot H. Cold induced injury to porcine corneal endothelial cells and its mediation by chelatable iron. Cornea 25: 68-77, 2006
66. Kerkweg U, Jakob M, de Groot H, Mannherz HG, Rauen U. Cold induced apoptosis of rat liver endothelial cells: contribution of mitochondrial alterations. Transplantation 76: 501-508, 2003
67. Molitoris BA, Sandoval R, Sutton TA. Endothelial injury and dysfunction in ischemic acute renal failure. Crit Care Med 30:S235-240, 2002
68. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation 63:968-974, 1997
69. Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 287: F181-187, 2004
70. Huang H, Salahudeen AK. Cold induces catalytic iron release of cytochrome P-450 origin: a critical step in cold storage induced renal injury. Am J Transplant 2: 631-639, 2002
71. Dragun D, Hoff U, Park JK, Qun Y, Schneider W, Luft FC, Haller H. Prolonged cold preservation augments vascular injury independent of renal transplant immunogenicity and function. Kidney Int 60:1173-1181, 2001
72. Jassem W, Koo DD, Cerundolo L, Rela M, Heaton ND, Fuggle SV. Cadeveric versus living-donor livers: differences in inflammatory markers after transplantation. Transplantation 76:1599-603, 2003
73. Troppmann C, Gillingham KJ, Benedetti E. Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. Transplantation 58:962-968, 1995
74. Koning OH, Ploeg RJ, van Bockel JH. Risk factors for delayed graft function in cadaveric kidney transplantation: A prospective study of renal function and graft survival after preservation with UW solution in multi-organ donors: European multicenter study group. Transplantation 15:1620-1628, 1997
75. Cosio FG, Qiu W, Henry ML et al. Factors related to the donor organ are major determinants of renal allograft function and survival. Transplantation 62:1571-1576, 1996
76. Entman ML, Smith CW. Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. Cardiovasc Res. 28:1301-1311, 1994
77. Fabre JW. The role of polymorphic donor peptides in allograft recognition and rejection. Immunol Rev. 154:21-43, 1996
78. Lefer AM. Role of selectins in myocardial ischemia-reperfusion injury. Ann Thorac Surg. 60:773-777, 1995
79. Paller MS. The cell biology of reperfusion injury in the kidney. J Investig Med. 42:632-639, 1995
80. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. Circulation 104:3158-3167, 2001
81. Miller MJ. Preconditioning for cardioprotection against ischemia reperfusion injury: the roles of nitric oxide, reactive oxygen species, heat shock proteins, reactive hyperemia and antioxidants-a mini review. Can J Cardiol 17:1075-1082, 2001
82. Liang BT, Jacobson KA. Adenosine and ischemic preconditioning. Curr Pharm Des 5:1029-1041, 1999
83. Schutte H, Witzenrath M, Mayer K, Rosseau S, Seeger W, Grimminger F. Short term "preconditioning" with inhaled nitric oxide protects rabbit lungs against ischemia-reperfusion injury. Transplantation 72:1363-1370, 2001
84. Ricciardi R, Schaffer BK, Kim RD et al. Protective effects of ischemic preconditioning on the cold-preserved liver are tyrosine kinase dependent. Transplantation 72:406-412, 2001
85. Sola A, De Oca J, Gonzalez R et al. Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. Ann Surg 234:98-106, 2001
86. Peralta C, Bartrons R, Serafin A et al. Adenosine monophosphate-activated protein kinase mediates the protective effect of ischemic preconditioning on hepatic ischemis-reperfusion injury in the rat. Hepatology 34:1164-1173, 2001
87. Guinzberg R, Cortés D, Díaz-Cruz A, Riveros-Rosas H, Villalobos-Molina R, Piña E. Inosine released after hypoxia activates hepatic glucose liberation through A3 adenosine receptors. Am J Physiol Endocrinol Metabol 290: E940-51, 2006

88. Dragun D, Lukitsch I, Tullius SG et al. Inhibition of intercellular adhesion molecule-1 with anti-sense deoxynucleotides prolongs renal isograft survival in the rat. Kidney Int. 54:2113-2122, 1998
89. Pratschke J, Kofla G, Wilhem MJ et al. Improvements in early behaviour of rat kidney allografts after treatment of the brain-dead donor. Ann Surg 234:732-740, 2001
90. Kusaka M, Zandi-Nejad K, Kato S et al. Exploitation of the continuum between early ischemia/reperfusion injury and host alloresponsiveness: indefinite kidney survival by treatment with a soluble P-selectin ligand and loe-dose cyclosporine in combination. Transplantation 67:1255-1261, 1999
91. Marubyashi S, Oshiro Y, Fukuma A, Okada K, Maeda T, Dohi K. Protective effect of monoclonal antibodies to adhesion molecules on rat liver ischemia-reperfusion injury. Transplant Proc 31:1054-1059, 1999
92. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141-179, 1994
93. Collins T, Read MA, Neish AS, Whiteley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF kappa B and cytokine-inducible enhances. FASEB J 9:899-909, 1995
94. Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA. Role of nuclear factor-kappa B in atherogenesis. Exp Physiol 82:297-304, 1997
95. Vos IHC, Govers R, Groene H-J et al. NF-kappa B decoy oligodeoxynucleotides reduce monocyte infiltration in renal allografts. FASEB J 14:815-822, 2000
96. Van der Woude FJ, Schnuelle P, Yard BA. Preconditioning strategies to limit graft immunogenicity and cold ischemic organ injury. J Investig Med. 52(5):323-329, 2004
97. Kuecuek O, Mantouvalou L, Klemz R et al. Significant reduction of proinflammatory cytokines by treatment of the brain-dead donor. Transplant Proc 37:387-388, 2005
98. Adembri C, Massagrande AM, Tani A et al. Carbamylated erythropoietin derivative is neuroprotective in an experimental model of traumatic brain injury. Crit Care Med 2008 in press
99. Wang Y, Zhang ZG, Rhodes K et al. Post-ischemic treatment with erythropoietin or carbamylated erythropoietin reduces infarction and improves neurological outcome in a rat model of focal cerebral ischemia. Br J Pharmacol 151: 1377-84, 2007
100. Leuvenink HG, van Goor H, Navis GJ. Carbamylated erythropoietin (CEPO) reduces renal inflammation during brain death (SA-FC112). J Am Soc Nephrol (Abstract issue) 16:106A, 2005
101. Amersi F, Buelow R, Kato H et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 104:1631-39, 1999
102. Forthenberry JD, Huber AR, Owens ML. Inotropes inhibit endothelial cell surface adhesion molecules induced by interleukin-1beta. Crit Care Med 25:303-8, 1997
103. Schnuelle P, Berger S, de Boer J, Persijn G, van der Woude FJ. Effects of catecholamine application to brain dead donors on graft survival in solid organ transplantation. Transplantation 72:455-463, 2001
104. Schnuelle P, Lorenz D, Mueller A, Trede M, van der Woude FJ. Donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. Kidney Int 56:738-746, 1999
105. Liu Z, Hoeger S, Schuelle P et al. Donor dopamine pre-treatment inhibits tubulitis in renal allografts subjected to prolonged cold preservation. Transplantation 83:297-303, 2007
106. Goettmann U, Notheisen A, Brinkkoetter PT et al. Influence of donor pre-treatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 79:1344-1350, 2005
107. Yard B, Beck G, schnuelle P et al. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. Am J Transplant 4:22-30, 2004
108. Brinkkoetter PT, Beck GC, Goettmann U et al. Hypothemia-induced loss of endothelial barrier function is restored after dopamine pre-treatment: role of p42/p44 activation. Transplantation 82:534-542, 2006
109. Kapper S, Beck G, Riedel S et al. Modulation of chemokine production and expression of adhesion molecules in renal tubular epithelial and endothelial cells by catecholamines. Transplantation 74: 253-260, 2002
110. Riese U, Brenner S, Docke WD et al. Catecholamines induces IL10 release in patients suffering from acute myocardial infarction by transactivating its promoter in monocytic but not in T cells. Mol Cell Biochem 212:45-50, 2000
111. Woiciechowsky C, Asadullah K, Estler D et al. Sympathetic activation triggers systemic IL10 release in immunodepression induced brain injury. Nat Med 4:808-813, 1998
112. Berger SP, Hunger M, Yard BA, Schnuelle P, van der Woude FJ. Dopamine induces the expression of heme oxygenase-1 by human endothelial cells in vitro. Kidney Int 58:2314-2319, 2000
113. Schnuelle P, Yard BA, Braun C et al. Impact of donor dopamine on immediate graft function after kidney transplantation. Am J Transplant. 4:419-426, 2004
114. Sammut IA, Foresti R, Clark JE et al. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. Br J Pharmacol 125:1437-1444, 1998

115. Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ, Winslow RM. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. Circ Res 83:568-577, 1998
116. Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S. Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 272:32804-32809, 1997
117. Durante W, Schafer AI. Carbon monoxide and vascular cell function. Int J Mol Med 2:255-262, 1998
118. Zhang X, Shan P, Otterbein LE et al. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J Biol Chem. 278:1248-1258, 2003
119. Otterbein LE, Bach FH, Alam J et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 6:422-428, 2000
120. Nakao A, Neto JS, Kanno S et al. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. Am J Transplant 5:282-291, 2005
121. Sikorski EM, Hock T, Hill-Kapturczak N, Agarwal A. The story so far: Molecular regulation of the heme oxygenase-1 gene in renal injury. Am J Physiol Renal Physiol 286:F425-441, 2004
122. Matsumoto M, Makino Y, Tanaka T et al. Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. J Am Soc Nephrol 14:1825-1832, 2003
123. Otterbein LE, Zuckerbraun BS, Haga M et al. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 9:183-190, 2003
124. Kaizu T, Nakao A, Tsung A et al. Carbon monoxide inhalation ameliorates cold-ischemia reperfusion injury after rat liver transplantation. Surgery 138:229-35, 2005
125. Kohmoto J, Nakao A, Kaizu T et al. Low-dose carbon monoxide inhalation prevents ischemia/reperfusion injury of transplanted rat lung grafts. Surgery 140:179-185, 2006
126. Nakao A, Toyokawa H, Tsung A et al. Ex vivo application of carbon monoxide in university of Wisconsin solution to prevent intestinal cold ischemia/reperfusion injury. Am J Transplant 6:2243-2255, 2006
127. Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, Green CJ. Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. Curr Pharm Des. 9:2525-2539, 2003
128. Clark JE, Naughton P, Shurey S et al. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ Res 93:e2-e8, 2003
129. Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R, Motterlini R. Treatment with CORMs during cold storage improves renal function at reperfusion. Kidney Int 69:239-247, 2006
130. Foresti R, Hammad J, Clark JE et al. Vasoactive properties of CORM-3, a novel water soluble carbon monoxide releasing molecule. Br J Pharmacol 142:453-460, 2004
131. Theruvath TP, Zhong Z, Currin RT, Ramshesh VK, Lemasters JJ. Endothelial nitric oxide synthase protects transplantated mouse livers against storage/reperfusion injury: Role of vasodilatory and innate immunity pathways. Transplant Proc 38:3351-3357, 2006
132. Giraldez RR, Panda A, Xia Y, Sanders SP, Zweier JL. Decreased nitric oxide synthase activity causes impaired endothelium dependent relaxation in the postischemic heart. J Biol Chem 272:21420-21426, 1997
133. Tripatara P, Patel NSA, Webb A et al. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: Role for xanthine oxidoreductase. J Am Soc Nephrol 18:570-580, 2007
134. Johansen D, Ytrehus K, Baxter GF. Exogenous hydrogen sulfide (H2S) protects against regional myocardial ischemia-reperfusion injury--Evidence for a role of K ATP channels. Basic Res Cardiol 101:53-60, 2006
135. Terasaki PI, Gjertson D, Cecka JM, Takemoto S.Fit and match hypothesis for kidney transplantation. Transplantation 62:441-445,1996
136. Opelz G, Wujciak T, Döhler B, Scherer S, Mytilineos J. HLA compatibility and organ survival. Collaborative Transplant Study. Rev. Immunogenet 1:334,1999

Chapter 2

Donor dopamine treatment limits pulmonary oedema and inflammation in lung allografts subjected to prolonged hypothermia

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Abstract

Endothelial barrier dysfunction severely compromises organ function after reperfusion. Since dopamine pre-treatment improves hypothermia-mediated barrier dysfunction, we tested the hypothesis that dopamine treatment of lung allografts positively affects tissue damage associated with hypothermic preservation and reperfusion.

Rats were treated for 1 hour with dopamine (5µg/min/kg) or vehicle (NaCl). Thereafter lungs were explanted, flushed with Perfadex-solution and stored at 4°C for different time periods. Peak inspiratory pressure (PIP), pulmonary arterial pressure (PAP) and lung weight were measured online during reperfusion. Inflammatory mediators in the perfusate and the expression of adhesion molecules in situ were measured after perfusion.

Lungs could tolerate a cold ischemia time (CIT) of up to 6 hours with stable PIP, PAP and no oedema formation upon reperfusion. CIT above 6 hours significantly increased PIP, PAP and pulmonary oedema in untreated but not in dopamine treated lungs ($p<0.001$ dopamine treated vs. untreated). Perfusion and ventilation alone induced a strong upregulation of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and adhesion molecules in untreated lungs, while in dopamine treated lungs significantly lower levels were found. Dopamine treatment also inhibited tissue damage associated with hypothermic preservation as measured by NADH staining.

Our study suggests that donor dopamine treatment is a highly effective modality to maintain organ quality of lung allograft. These findings are of high clinical relevance because prevention of tissue damage might reduce complications associated with lung transplantation and hence improve graft survival in lung transplant recipients.

Introduction

Implementation of lung transplantation as treatment modality for patients with end-stage lung disease became possible because of significant improvements in surgical techniques, organ preservation and immunosuppression. Nevertheless, cold preservation and ischemia/reperfusion (I/R) injury still represent a genuine problem that limits the success of lung transplantation. Both factors can provoke non-specific alveolar damage, lung oedema and hypoxemia (1). This significantly contributes to the high incidence of graft failure (2) and the poor one-year graft survival (3) in lung transplant recipients. Because graft failure remains the leading cause for morbidity and mortality after lung transplantation, the success of lung transplantation will critically depend on successfully reducing the incidence of graft failure.

In general, cold ischemia time (CIT) is considered to be an important factor for transplantation outcome, particularly in heart and lung transplant recipients (4). Deterioration in organ quality and function during cold ischemia is mediated by a series of events such as oxidative stress, iron release and intracellular calcium overload (4-6). This ultimately leads to cell death and hence to organ dysfunction.

Many clinical complications that are associated with prolonged CIT of lung allografts are caused by the loss of endothelial barrier function (7-10). Tissue oedema occurs as a consequence of barrier dysfunction and is associated with an increase in pulmonary arterial and inspiratory pressure. Because hypothermia also impairs the Na^+/K^+-ATPase (4, 5), fluid homeostasis is dysregulated in the alveolar space during reperfusion and hence oedema formation will aggravate (12). Apart from oedema formation, reperfusion induces an unspecific inflammatory response (13-16). Endothelial barrier dysfunction and leukocyte extravasation often occur together. Although barrier function might contribute to leukocyte extravasation it is at present unclear to what extent.

Previously, we could demonstrate *in vitro* that dopamine is not only able to prevent cold preservation-induced injury of endothelial cells but also promotes barrier function upon rewarming (5, 17). These effects were not receptor mediated and did not require *de novo* protein synthesis, but were likely dependent on the reactive oxygen species (ROS) scavenging properties of dopamine (5). Also clinical studies (18) and animal models (19) have demonstrated that donor dopamine pre-treatment beneficially affects immediate renal function and inflammation following warm and cold ischemia.

In the present study, we employed an isolated rat lung model to test the hypothesis that dopamine treatment of lung allografts positively affects tissue damage associated with hypothermic preservation and reperfusion.

Materials and Methods

Animals

Wistar rats weighing 300-350g were obtained from Janvier, Rennes, France. Animals were kept under standard conditions and fed standard rodent chow and water ad libitum. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and were approved by German federal regulations (RP Karlsruhe, AZ: 35-9185.81/G-66/04).

Anaesthesia and operative techniques

Animals were anesthetized by intraperitoneal injection of thiopental (60 mg/kg). One group of rats (n=9) received dopamine (5μg/min/kg body weight) intravenously for one hour. The dose was chosen on the basis of studies performed in a rat model for warm and cold ischemia (19, 20). Untreated rats served as control group (n=9). In both groups of animals a 14-gauge angiocatheter was inserted into the trachea by cervical tracheotomy. The animals were ventilated with a small animal respirator, using 95%O_2 / 5%CO_2 gas, a tidal volume of 2 ml and a rate of 60 breaths per min with 2cm H_2O of positive end-expiratory pressure. Median sternotomy and thymectomy were performed to expose the heart-lung block. After heparin injection into the right ventricle (1000U/kg), a canula was placed into the main pulmonary artery through the right ventricular outflow tract and secured with 3–0 braided silk sutures. The left atrium and ventricle were amputated to vent blood. A warm (37°C) modified Krebs-Henseleit-Buffer (NaCl: 118 mM, KCl: 4.7 mM, KH_2PO4: 1.2 mM, $NaHCO_3$: 24 mM, $MgSO_4$: 1.2 mM, glucose: 11.0 mM, $CaCl_2$: 1.7 mM and 2% bovine albumin), containing sodium bicarbonate to maintain the pH at 7.3 to 7.4 at 37°C, was used to perfuse the lung. The heart-lung block was mounted in a perfusion chamber and maintained at 37°C. Reperfusion was performed at a constant flow rate of 5ml/hr.

Experimental Protocol

Cold preservation was carried out by flushing the lungs with 20ml of cold (4°C) Perfadex solution through the main pulmonary artery. Thereafter, the heart-lung block was stored at

4°C for 4, 6 and 8 hours and subsequently reperfused at 37°C with warm Krebs-Henseleit-solution for different time periods. Lungs not subjected to hypothermic preservation were directly ventilated and perfused for similar time periods. All groups consisted of at least 9 animals. During reperfusion, lung oxygenation was assessed at 15, 30 60 90, 120 and 180 minutes. In each experiment the organ-oxygenation was stable (data not shown). Mean pulmonary arterial pressure (PAP) and pulmonary inspiratory pressure (PIP) were measured continuously and recorded every ten minutes (MCG, Hottinger-Baldwin-Messtechnik, Germany). Lung weight was recorded online to assess oedema formation. The extent of pulmonary oedema was also quantified by wet/dry weight (W/D) ratios. To this end, the middle right lung lobe was weighted directly before and after drying (72 hours at 70°C). Remaining lung tissue was frozen in liquid nitrogen and stored at –80°C or fixed in 4% (W/V) formaldehyde.

Immunohistology

At the end of all experiments, lungs were snap frozen in liquid nitrogen. Cryostat sections (3-5µm) were stained by an indirect immunoperoxidase technique. Briefly, ethanol-fixed sections were first incubated with PBS/BSA (5% w/v) and subsequently with 2% H_2O_2. Hereafter, the sections were incubated for 1 hour with ICAM or VCAM primary antibody (R&D Systems GmbH, Wiesbaden), followed by extensive washing and finally by incubation with a biotine-conjugated secondary IgG antibody for 1 hour. The sections were washed 6 times in PBS/BSA and incubated with streptavidin-HRP for 30 min. Antibody binding was visualized by diaminobenzidine using Vectastain. All sections were counterstained with hematoxylin-eosin. ICAM and VCAM were assessed semiquantitatively using a grading scale from - to +++ (-: negative, +: weakly positive, ++: positive, +++: strongly positive). More than 20 fields of view were blindly evaluated at 40 times.

Nicotinamide adenine dinucleotide (NADH) staining

The NADH staining was used to assess cellular viability after cold storage (8 hours) and warm reperfusion (3 hours). Staining was performed essentially the same as described by Gettman et al (21). In brief, cryostat sections (5 µm) were rinsed twice with 0.1 M phosphate buffered saline (PBS) pH 7.4 and subsequently incubated for 1 hr at 37ºC with nitro blue tetrazolium (NBT) solution and NADH (both from Sigma, St. Louis MO). For 100 ml of solution 25 ml NBT, 25 ml of 0.1 M PBS and 50 mg of NADH dissolved in distilled water was prepared. The reaction was terminated by rinsing the sections with cold PBS.

Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1)

CINC-1 concentration was measured in the perfusion solutions by ELISA (R&D Systems GmbH, Wiesbaden). Sensitivity of the ELISA was less than 0.08 pg/ml for CINC-1. Each experiment was performed at least five times and CINC-1 concentration for each individual sample was assessed in triplicate.

RNase Protection assay (RPA)

Total RNA was isolated from frozen lung tissue using Trizol reagent (Gibco BRL, Eggenstein, Germany). RPA was performed with the rCK1 cytokine Multi-Probe Template Set (riboQuant, PharMingen, Heidelberg, Germany) as recommended by the manufacturer. Briefly, a set of ^{32}P-labeled RNA probes were synthesized from DNA templates by T7 polymerase and hybridized overnight with 20µg of total RNA. Hereafter free probes and single-stranded RNA were digested with RNase H. The samples were loaded on a 5% denaturing polyacrylamide gel, dried and exposed to Omat MA Kodak film. The expression of each specific mRNA was quantified by densitometry. Two housekeeping genes, i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 were included in each experiment.

LightCycler Polymerase Chain Reaction (LC-PCR)

500 ng of total RNA was reversed transcribed into cDNA. C-DNA was diluted in 20 µl DEPC-treated water and stored at –20°C until analysis. Specific DNA standards were generated by PCR amplification of cDNA, purification of the amplified products, and quantification by spectrophotometry. LC-PCR of cDNA specimen and DNA standards were performed in a total volume of 20 µl, containing 2 µl FastStart DNA Master SYBR Green I, 10 pMol of gene-specific forward and reverse primers and 2 mM MgCl. The following primers were used: VCAM-1: forward: 5'CAGAGATTCAATTCAGTGGCCC3'; reverse: 5'TGAGACGGTCACCCTTGAACAG3', ELAM forward: 5'AGACTCCGGCATGTGGAATGA3'; reverse: 5'AACGCATTCACCACT3' and S16 forward: 5'CACCTAACCATACGCCTTGCTT3'; reverse: 5'TGGATCATAGGACAACTG3'. Before amplification, each sample was incubated for 2 minutes at 50°C followed by 5 minutes at 95°C. Amplification of VCAM and ELAM was perfused in 40 and 45 amplification cycles respectively. Each cycle consisted of denaturation for 15 seconds at 95°C, annealing for 1 minute at 55°C and extention for 1 minute at 72°C. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90% and 100%. Linearity of the assay could be

demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of S16. Each experiment was repeated 3 times.

Statistical analysis

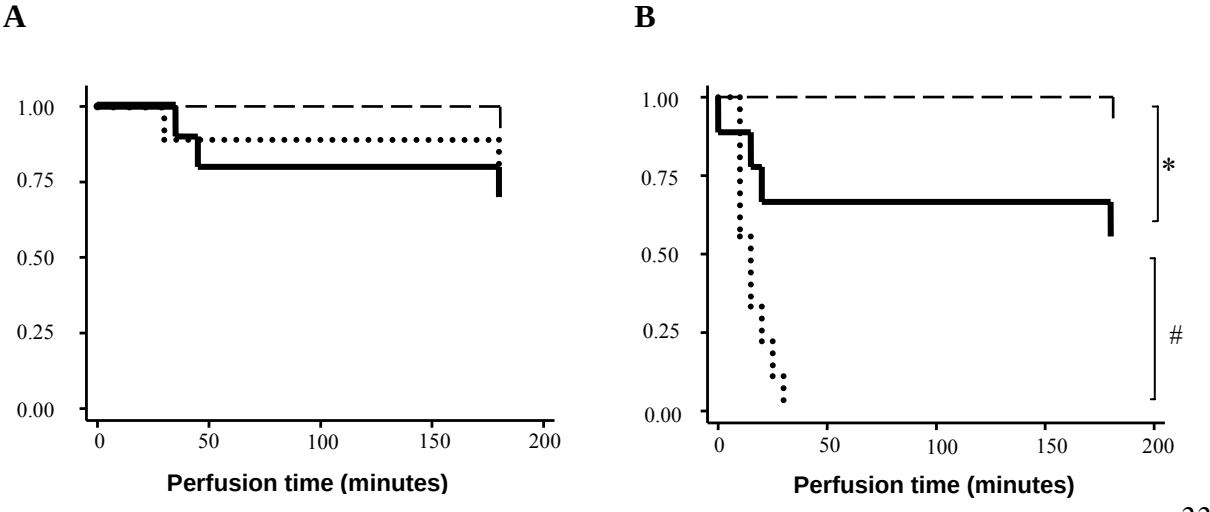
Data are shown as means ± SD. Comparisons were made using Wilcoxon's rank sum test, one-way ANOVA or the Mann-Whitney U test. Statistical analysis was performed using Stat View 4.01 (Abacus Corporation Inc., Berkeley, CA). A *p*-value of less than 0.05 was considered as significant.

Results

Influence of cold preservation on PAP, PIP and oedema formation during reperfusion

To investigate the influence of cold preservation on PAP, PIP and oedema formation, lungs from dopamine and vehicle treated rats were stored in Perfadex solution for 4, 6 and 8 hours, and subsequently perfused for 180 minutes with warm Krebs-Henseleit solution. Lungs not subjected to cold preservation were use for comparison. When cold preservation time was 4 and 6 hours, the increase in lung weight did not significantly differ between lungs that were subjected to cold preservation and lungs that were not. This was irrespective of dopamine treatment (Fig. 1A). However, in lungs that were preserved for 8 hours, lung weight increased more than 4g within 35 minutes in lungs obtained from vehicle treated rats (n=9). Dopamine pre-treatment significantly prolonged the time of perfusion in these lungs, as only in 4 out of 9 lungs weight increased more than 4g during the reperfusion period (Fig 1B, $p<0.003$, untreated versus dopamine treated).

Figure 1



C

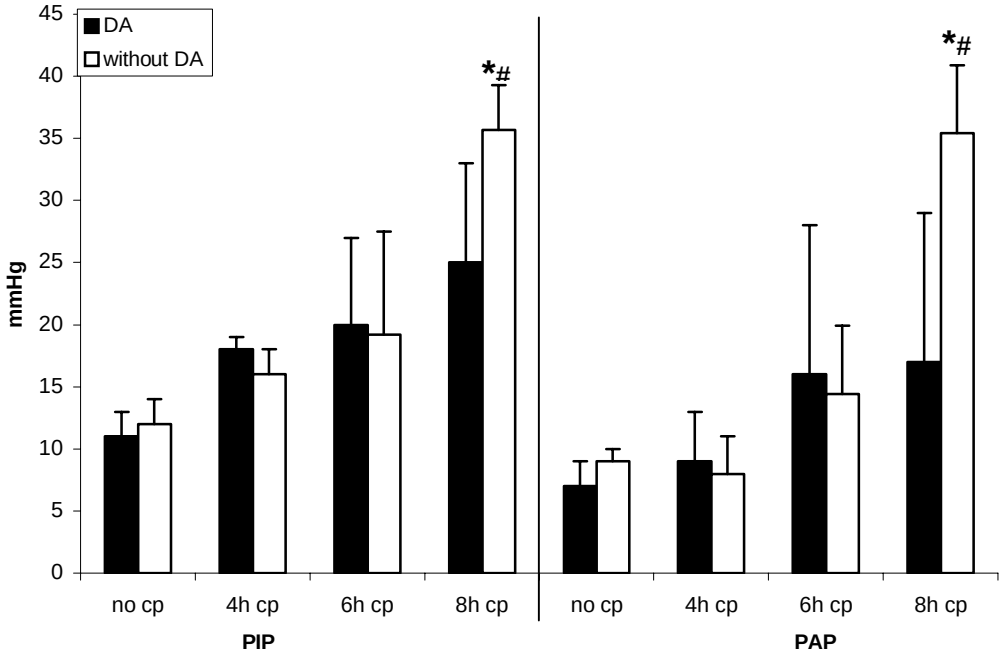


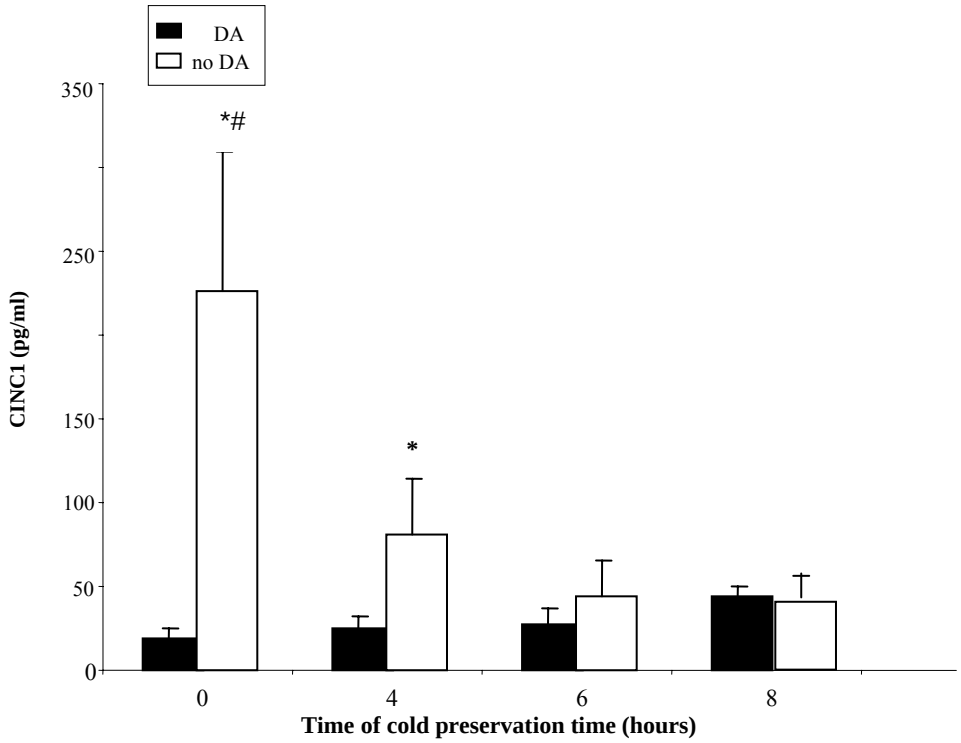
***Figure 1** (**A and B**) Influence of dopamine pre-treatment on pulmonary oedema formation. Rats were pre-treated with dopamine (5µg/min/kg body weight) 1 hr before lung explantation (n=9; bold line). Untreated rats (n=10; dotted line) served as control. The lungs were subjected to cold preservation for 6 hr (**A**) or 8 hr (**B**) and subsequently reperfused with warm Krebs-Henseleit solution for 180 minutes. Lungs not subjected to cold preservation (n=9; striped line) served as additional control Kaplan-Meyer analysis was performed using delta (Δ) weight increase more than 4g as endpoint. Significant differences between the groups were only observed after 8 hr of cold preservation: dopamine pre-treatment and cold preservation vs naïve and no cold preservation *: p<0.05, No dopamine pre-treatment vs dopamine pre-treatment #: p<0.01 (by log-rank-test). **C**: Influence of cold preservation time on peak inspiratory pressure (PIP) and pulmonary arterial pressure (PAP). Rats were pre-treated with dopamine (DA) (filled bars) as described in figure A and B or left untreated (open bars). Hereafter, the lungs were subjected to cold preservation for different time periods and subsequently ventilated and reperfused with warm Krebs-Henseleit solution for 180 minutes. Each group consisted of 9 animals. The results are expressed as mean PAP or PIP ± SD measured at the end of the reperfusion period. *: p<0.01 no cp vs. 8h cp, #: p<0.05 DA vs. without DA.*

Both, PIP and PAP also remained stable during 180 minutes of warm reperfusion in lungs that were preserved for 6 hours or less, but increased significantly when the preservation period lasted for 8 hours. Dopamine pre-treatment significantly blunted the increase in PIP and PAP during reperfusion (Fig 1C).

Influence of cold preservation on inflammatory mediators

Figure 2

A



B

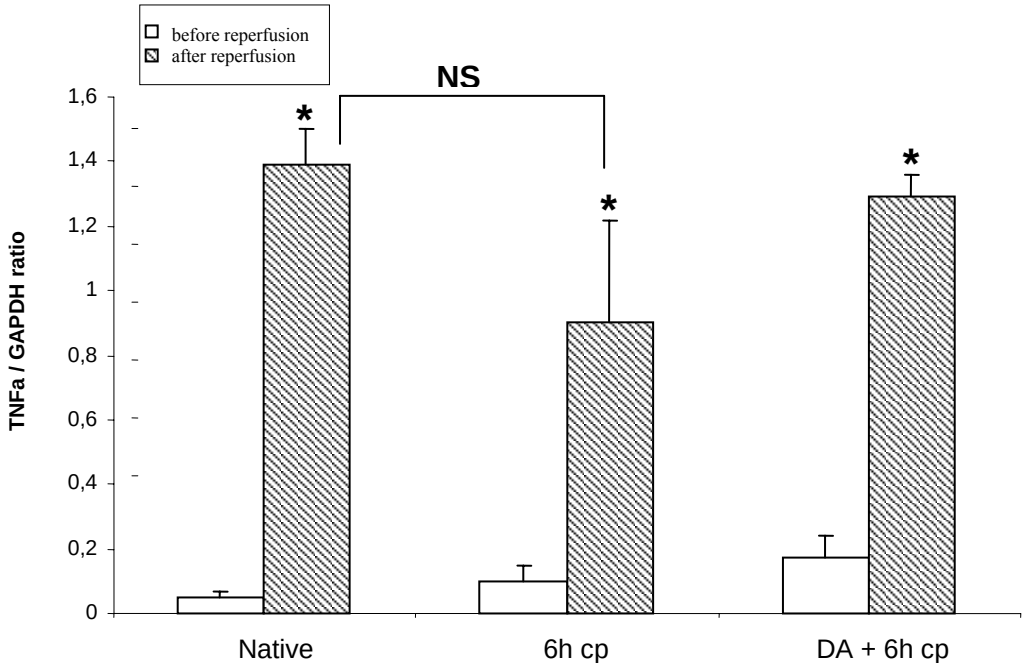


Figure 2 A*: Influence of cold preservation time on CINC-1 production. Rats were treated with dopamine (DA) (filled bars) as described in figure 1 or left untreated (no DA) (open bars). Hereafter the lungs were either directly ventilated and perfused with warm Krebs-Henseleit solution for 180 minutes or subjected to 4, 6 and 8 hr of cold preservation.*

*Immediately after cold preservation, the lungs were ventilated and perfused. CINC-1 concentration was measured in the perfusate solution at the end of the reperfusion period. Each group consisted of 5 animals. The results are expressed as mean CINC-1 (pg/ml) ± SD. * p<0.01 DA vs. no DA, #: p<0.01 0 hours vs. 6 hr of cold preservation.* ***B****: Influence of dopamine treatment on TNFα mRNA expression. Total RNA was isolated from native lungs (n=5), from lungs, with 6 hr of cold preservation (6h cp) and from dopamine pre-treated lungs, also subjected to 6 hr of cold preservation (DA + 6h cp). RNA was either isolated before (open bars) or after ventilation and warm reperfusion (180 minutes). TNFα mRNA expression was measured by means of RPA and standardized for equal expression of GAPDH as described in materials All groups consisted of 6 animals. The results are expressed as mean TNFα/GAPDH ratio ± SD. * p<0.01 before vs. after reperfusion.*

To study if cold preservation might affect the inflammatory response after reperfusion, we assessed the production of the neutrophil chemotactic protein CINC-1 in the perfusate of lungs. In addition we tested, if dopamine pre-treatment influences CINC-1 production. In naïve lungs not subjected to cold storage, CINC-1 was clearly present in the perfusate 3 hours after perfusion. This was significantly inhibited by dopamine pre-treatment. In lungs from vehicle treated rats, CINC-1 production inversely correlated with the time of cold preservation. The production of CINC-1 in dopamine treated lungs remained low, even after prolonged 8 hours of cold preservation (Fig 2A). To assess the influence of cold preservation and dopamine treatment on other inflammatory mediators, a cytokine multi-probe RNAse protection assay was used. After reperfusion TNF α mRNA expression was upregulated. This was neither influenced by cold preservation time nor by dopamine pre-treatment (Fig. 2B). Similar results were found for IL-1α, IL-1β and IL-6 (data not shown).

Similar as CINC-1 production, mRNA expression of VCAM-1 and ELAM were upregulated during warm reperfusion, only in lungs that were not subjected to cold preservation. Dopamine pre-treatment significantly inhibited the upregulation of these mRNA`s. Upregulation of VCAM and ELAM mRNA during reperfusion was less pronounced after cold preservation (Fig. 3). Immunhistological staining for ICAM-1 and VCAM-1 confirmed that upregulation of adhesion molecules was clearly more pronounced in naïve lungs, not subjected to cold preservation (Fig. 4 and Table 1).

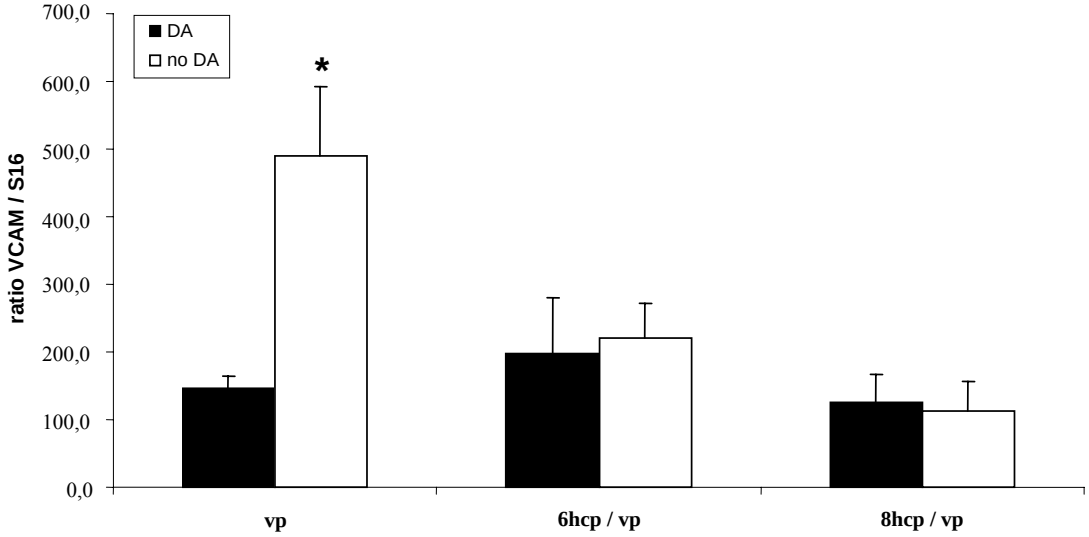
Tissue viability after cold preservation and warm reperfusion

To study tissue viability after cold storage and reperfusion and if this was influenced by dopamine pre-treatment, we made use of the NADH histological staining. Although both lungs obtained from untreated and dopamine treated rats clearly showed a positive NADH staining after cold storage and subsequent 3 hours of warm reperfusion, the staining intensity

of the latter lungs was more prominent (Fig 5). Thus, suggesting that tissue viability was improved by dopamine treatment in this model.

Figure 3

A



B

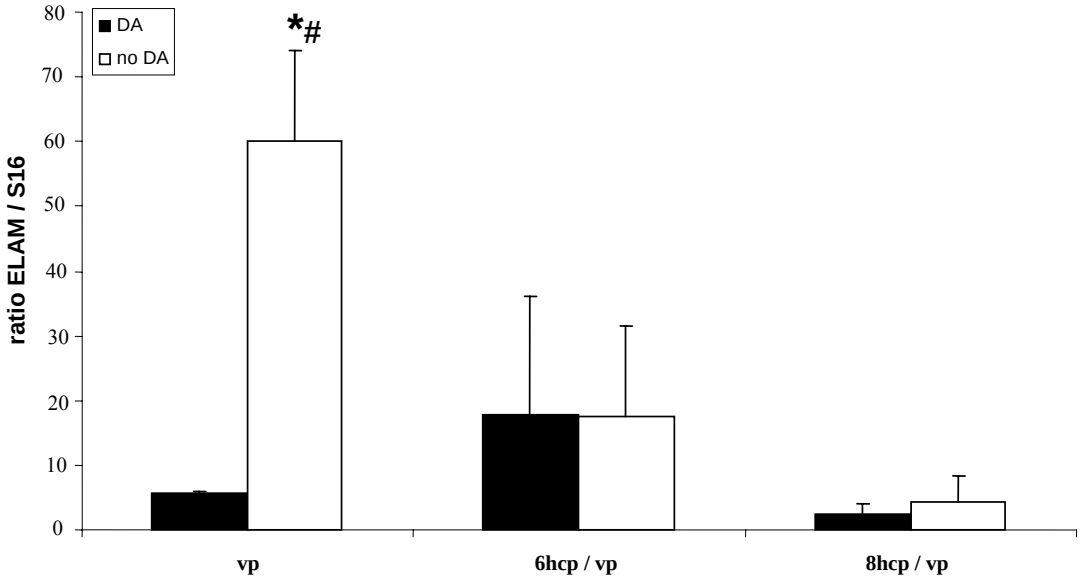


Figure 3 *Influence of dopamine treatment on VCAM-1 and ELAM-1 mRNA expression. Rats were treated with dopamine (DA) (filled bars) as described in figure 1 or left untreated (no DA) (open bars). Hereafter the lungs were either directly ventilated and perfused (vp) with warm Krebs-Henseleit solution for 180 minutes or subjected to 6 or 8 hr of cold preservation (6hcp and 8hcp respectively). Immediately after cold preservation, the lungs were ventilated and perfused for 180 minutes. Total RNA was isolated from the tissue and assessed for VCAM-1 (**A**) and ELAM-1 (**B**) mRNA expression by LightCycler PCR. All samples were*

*normalized for equal S16 mRNA expression. Each group consisted of 5 animals. The results are expressed as mean adhesion molecule/S16 ratio ± SD. * p<0.01 DA vs. no DA, #: p<0.05 0 hours vs. 6 hours of cold preservation.*

Figure 4

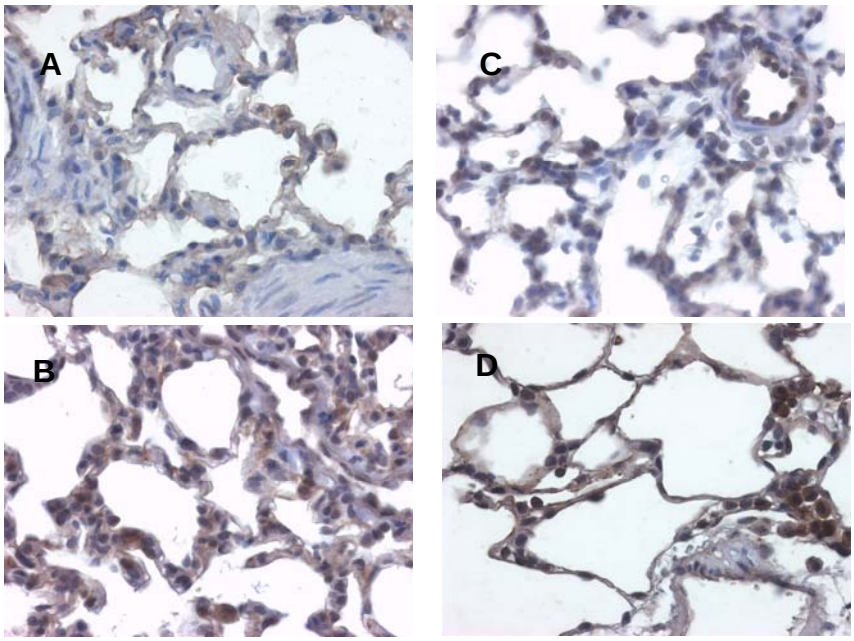


Figure 4 *Influence of dopamine treatment on ICAM expression. Rats were treated as described in Figure 1, with dopamine (A and C) or left untreated (B and D). Hereafter the lungs were directly reperfused and ventilated for 3 hr (A and B) or subjected to 8 hr of cold preservation before reperfusion and reventilation (3hr) was initiated. The results of a representative experiment (n=5) is depicted. Original magnification was X40.*

Table 1 Immunohistological staining for ICAM-1 and VCAM-1 in lung tissue

		ICAM		VCAM	
		+ DA	- DA	+ DA	- DA
Native [a]		+	+ [b]	-	-
No cold preservation [c]		+	+++	++	+++
Cold preservation [c]	6 hr	++	++	++	+++
	8 hr	++	++	++	+++

[a]: no cold preservation and no reperfusion, [b]: results are expressed semi-quantitatively: (-: negative, +: slightly positive, ++: positive, +++: strongly positive), [c]:analysis after 3 hr of warm reperfusion.

Figure 5

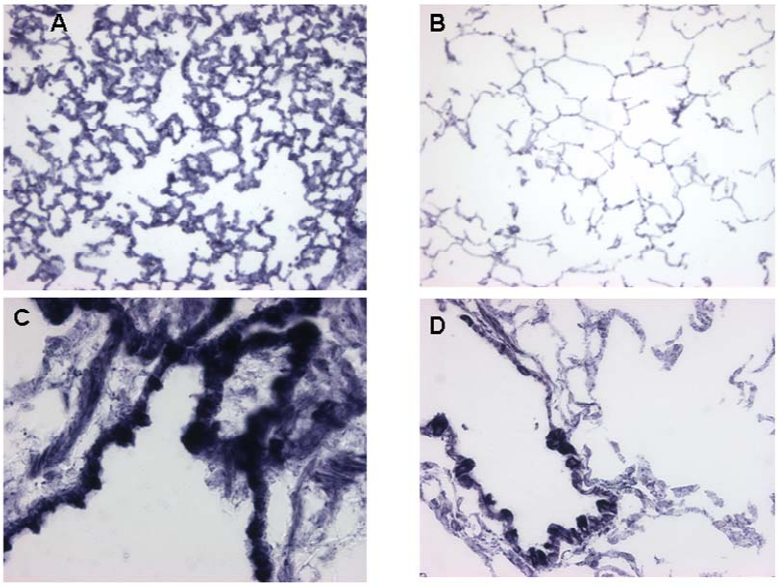


Figure 5 *Influence of dopamine treatment on tissue viability. Rats were treated with dopamine (A and C) or left untreated (B and D) as described in figure 1. The lungs were explanted and subjected to 8hr of cold preservation. NADH staining of lung tissue was performed after 180 minutes of warm reperfusion. Note a more intensive NADH staining in dopamine treated lungs. The results of a representative experiment (n=5) is depicted. Original magnification was X10 in A and B and X40 in C and D.*

Discussion

In general, CIT negatively affects graft function and long-term graft survival after organ transplantation (22). As CIT increases, the quality of allografts deteriorates (22), which, in view of the danger hypothesis, might result in increased tissue immunogenicity. Since we previously could show that donor dopamine usage significantly decreased the incidence of delayed graft function after renal transplantation (20, 23, 24) and that dopamine improves endothelial barrier function after cold storage (17), we tested in the present study the hypothesis that dopamine treatment of lung allografts positively affects tissue damage associated with hypothermic preservation and reperfusion.

The major findings of this study are: first, dopamine pre-treatment inhibits oedema formation and positively affects PAP and PIP, after cold preservation and warm reperfusion. Second, dopamine pre-treatment abrogates CINC-1 production during lung reperfusion. Third, dopamine pre-treatment inhibits the upregulation of adhesion molecules, i.e. ICAM-1. VCAM-1, ELAM,

during reperfusion, but it does not affect the production of TNFα, IL-1α, IL-1β and IL-6. Fourth, tissue viability is better maintained in dopamine pre-treated lungs after cold storage and reperfusion.

Pulmonary oedema (PE) is a common problem in both organ donors and lung transplant recipients, contributing significantly to poor donor lung function (25). Implementation of donor management strategies to improve fluid handling of the lung, might therefore significantly affect immediate graft function and graft survival after lung transplantation. Our data on prevention of PE by dopamine pre-treatment are compatible with the findings of Ware et al (26) who showed that preharvest administration of dopamine in low to moderate doses was associated with faster alveolar fluid clearance and to a lower wet-to-dry ratio. These findings also imply that dopamine usage in donor management should not only be restricted to hemodynamic instable donors.

The Na^+/K^+-ATPase is ubiquitously expressed and is essential for maintenance of membrane potential and cell volume (27) In addition, it is also implicated in lung oedema clearance by increasing active Na^+ transport (28). Since prolonged cold preservation of lung allografts result in severe inhibition of the Na^+/K^+-ATPase (29), unsurprisingly, our data show that a cold preservation time beyond 6 hours results in profound oedema formation. Although dopamine inhibits the Na^+/K^+-ATPase activity in most tissues, e.g. brain (30) and kidney (31), there is also evidence that it increases the ability of the lungs to clear oedema via regulation of the alveolar epithelial Na^+/K^+-ATPase function (32, 33). This might explain our findings that pre-treatment of lungs with dopamine reduced PE during warm reperfusion in our model. Since PAP and PIP will increase as a consequence of PE, it is clear that dopamine also beneficially affects these parameters.

In addition to the role of Na^+/K^+-ATPase on PE, endothelial barrier dysfunction is likely involved in this process. An increase in F-actin stress fibres, concomitantly with redistribution of adherence- and tight-junction proteins, leads to an imbalance between contractile and tethering forces in favor of barrier dysfunction (34). As the proper function of adherence- and tight-junction proteins is ATP dependent, cold preservation is an eligible condition for barrier dysfunction because cold preservation leads to ATP depletion (4). Inasmuch as in vitro studies show that dopamine pre-treatment cannot completely prevent redistribution of junctional proteins during hypothermia of endothelial cells, it does restore normal distribution of ZO-1 and VE-cadherin and barrier function upon rewarming (17). Furthermore, dopamine and structurally related molecules have the propensity to protect endothelial cells for necrosis during cold preservation (5).Therefore, prevention of endothelial damage and restoration of barrier function might both contribute to the reduction of PE in dopamine pre-treated lungs.

I/R injury is histological characterized by an inflammatory response, reflected by an increase in chemokine production, upregulation of adhesion molecules and production of pro-inflammatory cytokines. De Perrot et al (35) have demonstrated that IL-8, released early during reperfusion, predicts graft function in human lung transplantation. In our model, dopamine pre-treatment inhibits CINC-1 production during reperfusion, a rat homologue for human IL-8. CINC-1 production was also reduced by cold preservation in a time dependent fashion. These findings are compatible with previous studies from our group using the same model (36) and with in vitro studies on renal epithelial cells (37). Although NADH staining is questionable to assess tissue viability, as false positive staining might occur (38), negative NADH staining is in general consistent with tissue non-viability. Because we observed a clear reduction in NADH staining after cold preservation, this might indicate that tissue viability is decreased in these lungs. The observed reduction in CINC-1 production after cold storage could therefore be due to tissue damage.

In contrast to CINC-1 production, dopamine did not influence the upregulation of TNFα mRNA expression during reperfusion. The increased TNFα expression is likely originating from alveolar macrophages as was demonstrated by Zhao et al (39), Leo et al (40) and Ashish et al (41). Activated alveolar macrophages, in turn, can modulate the inflammatory response by activating alveolar epithelial cells (41). Although dopamine did not influence TNFα mRNA expression, the expression of ICAM-1, VCAM-1 and ELAM was decreased by dopamine. Since dopamine delays TNFα mediated upregulation of adhesion molecules (37), this might explain why a reduction in the expression of ICAM-1, VCAM-1 and ELAM was found in dopamine treated lungs despite no reduction in TNFα mRNA expression.

In conclusion, our study demonstrates that dopamine pre-treatment significantly limits formation of PE during reperfusion of lungs, subjected to prolonged cold preservation. Moreover, dopamine might act as anti-inflammatory drug under these conditions, as it inhibits CINC-1 production and the expression of adhesion molecules. Since poor immediate graft function is frequently observed in lung transplant recipients, strategies to maintain donor lung function are warranted. Donor dopamine usage has already proven to be beneficial after renal transplantation (23, 24) and seems to be an auspicious treatment modality to maintain organ quality of lung allografts after hypothermic preservation.

References:

1. Hidalgo MA, Sarathchandra P, Fryer RR et al. Scanning electron microscopic changes in morphology of pulmonary endothelium in rat lung isografts following hypothermic ischemic storage and transplantation. Int J Exp Pathol 1995; 76: 339.
2. Whitson BA, Nath DS, Johnson AC et al. Risk factors for primary graft dysfunction after lung transplantation. J Thorac Cardiovasc Surg. 2006; 131 (1): 73-80.
3. Mal H, Brugiere O, Dauriat G et al. Lung transplantation in patients with pulmonary fibrosis. Rev Pneumol Clin. 2005; 61 (3): 232-8.
4. De Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med. 2003; 167 (4): 490-511.
5. Yard B, Beck G, Schnuelle P, Braun C, van der Woude FJ. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. Am J Transpl 2004; 4: 22-30.
6. Ikeyama K, Sakai H, Omasa M et al. Effects of cold preservation in the lung mechanical properties in rats. Eur Surg Res 2005; 37 (2): 85-91.
7. Hidalgo MA, Shah KA, Fuller BJ et al. Cold ischemia-induced damage to vascular endothelium results in permeability alterations in transplanted lungs. J Thorac Cardiovasc Surg 1996; 112:1027.
8. Carbognani P, Spaggiari L, Rusca M et al. Pulmonary endothelial cell modifications after cold storage in solid organ preservation solutions. J Int Med Res 1995; 23: 200-206.
9. Pickford MA, Manek S, Green CJ et al. The pathology of rat lung isografts following 48 or 72-hour cold storage and subsequent reperfusion in vivo for up to 1 month. Int J Exp Pathol 1992; 73: 685-697.
10. Hall SM, Odom N, McGregor CG et al. Transient ultrastructural injury and repair of pulmonary capillaries in transplantated rat lung: effect of preservation and reperfusion. Am J Respir Cell Mol Biol 1992; 7:49.
11. Ware LB, Golden JA, Finkbeiner WE, Matthay Ma. Alveolar epithelial fluid transport capacity in reperfusion lung injury after lung transplantation. Am J Respir Crit Care Med. 1999; 159: 980-988.
12. Wang LS, Yoshikawa K, Miyoshi A et al. The effect of ischemic time and temperature on lung preservation in a simple ex vivo rabbit model used for functional assessment. J Thorac Cardiovasc Surg 1989; 98: 333-342.
13. Lemay S, Rabb H, Postlrt G; Singh AK. Prominent and substained upregulation of gp130-signaling cytokines and the chemokine MIP-2 in murine renal ischemia-reperfusion injury. Transplantation 2000; 69: 959-963.
14. Gerlach J, Jorres A, Nohr R, Zeilinger K, Spatkowski G, Neuhaus P. Local liberation of cytokines during liver preservation. Transpl Int 1999; 12: 261-265.
15. Oz MC, Liao H, Naka Y et al. Ischemia-induced interleukin-8 release after human heart transplantation: a potential role for endothelial cells. Circulation 1995; 92: II 428-II432.
16. Serrick C, Adoumie R, Giaid A, Shennib H. The early release of interleukin-2, tumor necrosis factor-alpha and interferon-gamma after ischemia reperfusion injury in the lung allograft. Transplantation 1994; 58: 1158-1162.
17. Brinkkoetter PT, Beck GC, Gottmann U et al. Hypothermia-induced loss of endothelial barrier function is restored after Dopamine pre-treatment: role of p42/p44 activation. Transplantation 2006; 82 (4): 534-542.
18. Schnuelle P, Berger S, de Boer J, Persijn G, van der Woude FJ. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. Transplantation 2001; 72(3): 455-63.
19. Goettmann U, Notheisen A, Braun C et al. Influence of donor pre-treatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 2005; 79: 1344-1350.
20. Goettmann U, Brinkkoetter PT, Braun C et al. Effect of pre-treatment with catecholamines on cold preservation and ischemia/reperfusion-injury in rats. Kidney Int. 2006; 70(2): 321-8.
21. Gettman MT, Lotan Y, Corwin TS et al. Radiofrequency coagulation of renal parenchyma: comparison of effects of energy generators on treatment efficacy. J Endourol 2002; 16: 83-88.
22. Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 2004; 287(2): F181.
23. Schnuelle P, Lorenz D, Mueller A et al. Donor catecholamineuse reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. Kidney Int 1999; 56(2): 738.
24. Schnuelle P, Berger S, de Boer Jet al. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. Transplantation 2001; 72(3): 455-63.
25. McCowin M, Hall TS, Babcock W, Solinger LL, Hall KW, Jablons D. The impact of changes in radiographic abnormalities in organ donors on successful lung donation. J Heart Lung transplant 2001; 20(2):242-243.
26. Ware LB, Fang X, Wang Y, Sakuma T, Hall TS, Matthay MA. Selected contribution: mechanisms that may stimulate the resolution of alveolar oedema in the transplantated human lung. J Appl Physiol 2002; 93(5):1869-1874.
27. Lingrel JB, Kuntzweiler T. Na+,K(+)-ATPase. J Biol Chem 1994; 269(31): 19659-19662.
28. Olivera W, Ridge K, Wood LD, Sznajder JI. Active sodium transport and alveolar epithelial Na-K-ATPase increase during subacute hyperoxia in rats. Am J Physiol 1994; 266:L577-L584.
29. Kim JD, Baker CJ, Danto SI, Stames VA, Barr ML. Modulation of pulmonary NA+ pump gene expression during cold storage and reperfusion. Transplantation 2000; 70(7): 1016-20.

30. Bertorello AM, Hopfield JF, Aperia A, Greengard P. Inhibition by dopamine of Na+-K+-ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. Nature 1990; 347(6291): 386-388.
31. Bertorello A, Aperia A. Inhibition of proximal tubule Na+-K+-AtPase activity requires simultaneous activation of DA1 and DA2 receptors. Am J Physiol 1990; 259: F924-F928.
32. Barnard ML, Olivera WG, Rutschmann DM, Bertorello AM, Katz AI, Snajder JI. Dopamine stimulates sodium transport and liquid clearance in rat epithelium. Am J Respir Crit Care Med 1997; 156: 709-714.
33. Saldias FJ, Lecuona E, Comellas AP, Ridge KM, Snajder IL. Dopamine restores lung ability to clear oedema in rats exposed to hyperoxia. Am J Respir Crit Care Med 1999; 159(2): 626-633.
34. Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol 2001; 91(4): 1487-500.
35. De Perrot M, Sekine Y, Fischer S et al. Interleukin-8 release during early reperfusion predicts graft function in human lung transplantation. Am J Respir Crit Care Med 2002; 165: 211-215.
36. Hanusch C, Nowak K, Gill I et al. Hypothermic preservation of lung allograft inhibits CINC-1, ELAM, VCAM-1 and ICAM-1 expression. Clin Exp Immunol 2007; 149: 364-371.
37. Kapper S, Beck G, Riedel S et al. Modulation of chemokine production and expression of adhesion molecules in renal tubular epithelial and endothelial cells by catecholamines. Transplantation 2002; 74(2):253.
38. Stern JM, Anderson JK, Lotan Y, et al. Nicotinamide adenine dinucleotide staining immediately following radio frequency ablation of renal tumors-is a positive stain synonymous with ablative failure? J Urol 2006; 176(5): 1969-72.
39. Zhao M, Fernandez LG, Doctor A, et al. Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. Am J Physiol Lung Cell Mol Physiol 2006; 291: L1018-1026.
40. Gazoni LM, Tribble CG, Zhao MQ, et al. Pulmonary macrophage inhibition and inhaled nitric oxide attenuate lung ischemia-reperfusion injury. Ann Thorac Surg 2007; 84: 247-253.
41. Sharma AK, Fernandez LG, Awad AS, et al. Proinflammatory response of alveolar epithelial cells is enhanced by alveolar macrophage-produced TNF-α during pulmonary ischemia-reperfusion injury. Am J Physiol Lung Cell Mol Physiol 2007; 293: L105-L113.

Chapter 3

Hypothermic injury: the mitochondrial calcium, ATP and ROS love-hate triangle out of balance.

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Abstract

Catecholamines prevent hypothermic cell death which accounts for severe tissue damage and impaired allograft function after prolonged organ preservation. Here, we identified cellular processes which govern hypothermia-mediated cell death in endothelial cells and how they are influenced by dopamine.

Lactate dehydrogenase assay, intracellular ATP, reactive oxygen species and reduced thio-group measurement, intracellular calcium measurement and mitochondrial calcium staining were performed in the study.

Intracellular ATP was almost completely depleted within 12 hrs of hypothermic preservation in untreated human umbilical vein endothelial cells (HUVEC), while dopamine pre-treatment significantly delayed ATP depletion. 4 hrs after hypothermia a redox imbalance was observed in untreated cells, which increased with the duration of hypothermia. The redox imbalance was primarily caused by depletion of SH reduction equivalents and was significantly inhibited by dopamine. In addition, hypothermia-induced Ca^{2+} influx and mitochondrial Ca^{2+} accumulation were both prevented by dopamine. The protective effect of dopamine was abrogated by ionomycin and sodium azide and partly by oligomycin and CCCP.

Our data demonstrated that loss of intracellular ATP, generation of a redox imbalance and accumulation of intracellular Ca^{2+} underlie cold preservation injury. Dopamine improves the redox balance, prevents intracellular Ca^{2+} accumulation and delays ATP depletion.

Introduction

Mitochondria are the major sites of ATP synthesis in eukaryotes. ATP is generated via oxidative phosphorylation (ox-phos), in which electrons are liberated from reduced substrates and finally delivered to O_2. Approximately 2% of consumed oxygen is converted into reactive oxygen species (ROS). During ox-phos, the respiratory chain complexes (complex I-IV) establish an H^+ gradient, which is then utilized to drive ATP synthesis via complex V, i.e. ATP synthase. ROS production ensues from the activity of the respiratory chain. The superoxide anion is produced at two major sites in the respiratory chain, i.e. in the ubiquinone- (Q) cycle via the ubisemiquinone radical intermediate (1, 2) and in complex I through a backward electron transfer from succinate to NAD^+ (3). Manganese superoxide dismutase (MnSOD) converts superoxide into hydrogenperoxide which, in turn, is reduced to hydroxyl radicals by Fenton chemistry (4). Apart from their role in ATP synthesis, mitochondria also play a pivotal role in other cellular processes such as calcium (Ca^{2+}) homeostasis (5, 6).

Under physiological conditions mitochondrial Ca^{2+}, ATP synthesis and ROS production are tightly regulated in a discrete balance, designated as the love-hate triangle (7). Ca^{2+} is a physiological stimulator of ox-phos to balance mitochondrial ATP output and cellular ATP demand (8-10). However, when mitochondrial Ca^{2+} overload occurs, Ca^{2+} becomes patho-physiologically relevant and is embodied in the regulation of opening of the mitochondrial permeability transition (MPT) pore. Opening of the MPT pore is mediated by high mitochondrial Ca^{2+} concentrations $[Ca^{2+}]_m$ and is facilitated by ROS. The mitochondrial membrane potential completely dissipates at a high open probability (P_O) of the MPT pore. Unstable membrane potential and redox transitions can occur under a variety of pathological conditions, e.g. ischemia/reperfusion injury, and might have negative consequences for mitochondrial integrity and cellular survival (11, 12).

Hypothermia and ischemia are two major hurdles in hypothermic organ preservation as they negatively affect mitochondrial function. Although it has been acknowledged that prolonged hypothermic preservation of organ allografts is detrimental to organ viability (13), the cellular events leading to hypothermic injury have not been elucidated completely. Nevertheless, various studies have highlighted the importance of ROS production, impairment of Ca^{2+} homeostasis and ATP depletion in hypothermic injury (14, 15).

In order to handle cold ischemic conditions, hibernating mammals have developed a number of strategies to limit the problems of Ca^{2+} homeostasis, ATP synthesis and ROS production. A

decreased ox-phos (16), the expression of uncoupling proteins in brown adipose tissue (17), as well as an increased Ca^{2+} uptake in the sarco-/ endoplasmic reticulum (18, 19) are amongst mechanisms used by hibernating mammals to adapt to long periods of hypothermia and reduced oxygen pressure.

We and others have demonstrated that dihydroxyphenolic compounds (20-22) and iron chelators (23, 24) can protect endothelial- and tubular epithelial cells against hypothermia mediated cell death. In the present study we hypothesized that these compounds mediate protection by balancing the mitochondrial Ca^{2+} - ATP - ROS love-hate triangle during hypothermia.

Materials and methods

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously (25). The cells were cultured in endothelial cell growth medium (EGM) (Promocell, Heidelberg, Germany) in T25 flasks (Greiner, Frickenhausen, Germany) coated with gelatine (1%). Confluent monolayers were passaged by Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO). Characterization of endothelial cells was performed on the basis of a positive uptake of acetylated LDL, Factor VIII related antigen and PECAM (CD31) expression, and a negative staining for alpha smooth muscle actin. All experiments were carried out at 100% confluence.

Dopamine treatment

HUVEC were incubated for 2hrs with 25µM of dopamine (DA, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). The culture flaks were washed 3 times with 1ml of PBS (Invitrogen, Karlsruhe, Germany) and stored for 24hrs at 4°C in HTK-, UW-solution or phenol-red free medium (Promocell). Since there was no difference between the use of the preservation solutions or phenol red free medium with respect to cell damage after hypothermic preservation, in all experiments described in this paper, the cells were stored in phenol-red free medium. In some experiments EDTA, BAPTA-AM, thapsigargin, myxothiazole, Na-azide, oligomycin, ionomycin, CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (all from Sigma-Aldrich, St. Louis, MO) were added to the medium during hypothermia.

LDH assay

Lactate dehydrogenase assays were performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, HUVEC were plated in 24-well plates, grown until confluence and stimulated with 25µM dopamine (DA) for 2hrs. The plates were washed 3 times with 1ml of PBS and stored for 24hrs at 4°C in phenol-red free medium. A 100µl aliquot of each supernatant was used to determine LDH release. In each experiment 100µl of phenol-red free medium was used as blank. The results are expressed as OD490nm, corrected for the blank value.

Measurement of reactive oxygen species

The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-H_2-DCFDA (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The formation of superoxide anions and hydroxyl radicals can both be assessed by this method. HUVEC grown in 96-well plates were pre-treated with 25µM DA for 2hrs. Then the cells were loaded with 10µM of CM- H_2-DCFDA for 45min at 37°C. The cells were incubated at 37°C and 4°C respectively and analysed by serial measurements in a Spectra Fluor Plus Fluorescent 96-well plate reader (Tecan Deutschland, Crailsdorf, Germany).

Assessment of SH reduction equivalents

Intracellular "-SH groups" were quantified with a standard assay based on the reduction of 5,5' Dithiobis-2-Nitrobenzoic acid (DTNB, Sigma). Cells were lysed in 10mM Tris-buffer w/ 1% Triton X-100 (Sigma), followed by centrifugation at 15,000rpm for 10 minutes. The supernatant was incubated with phosphate buffer containing DTNB at room temperature for 60 minutes. The quantification was conducted at 412nm using a spectrophotometer (Spectra Fluor Plus 96-well plate reader, Tecan).

Assessment of intracellular ATP

Confluent HUVEC monolayers were treated for 2hrs with 25µM dopamine or left untreated. Subsequently the cells were washed and stored at 4°C. Intracellular ATP was extracted at serial time points and measured by luciferase driven bioluminescence using the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany).

Confocal microscopy for mitochondrial calcium staining

Reduced Rhod-2 AM (Invitrogen, Karlsruhe, Germany) was used to detect mitochondrial calcium levels. Rhod-2 AM stock solution dissolved in DMSO (Sigma) was incubated with Na-borohydride ($NaBH_4$, Sigma) solution for 10 minutes at room temperature. HUVECs cultured on coverslips were incubated with phenol-red free medium containing 5μM dihydroRhod-2 AM at 37°C for 40 minutes. The cells were washed with PBY twice and subjected to 4°C**.** In some cases cells were simultaneously co-incubated with MitoTracker Green (200nM, Invitrogen, Karlsruhe, Germany). Fluorescence images were obtained by confocal microscopy (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany) under corresponding excitation and emission wave lengths. Quantification was performed by image analysis (Leica confocal software) and the results were expressed as relative levels of fluorescence intensity.

Measurements of intracellular Ca^{2+} concentrations

Measurements of intracellular Ca^{2+} concentrations were performed according to Koppel et al. (26). HUVEC were incubated with phenol-red free medium containing 4μm Fura-2 AM (Invitrogen, Karlsruhe, Germany) at 37°C for 1hr. The cells were washed with PBS twice and treated with dopamine for 2hrs. After washing the cells with PBS twice, phenol-red free media was added and the cells were subjected to 4°C for 3hrs. Coverslips were then mounted into a thermostatically regulated microscope chamber. A Zeiss Axiovert 35 (Zeiss, Hanau, Germany) inverted fluorescence microscope, equipped with a fluor 40/1.30 oil immersion objective and a charge-coupled device imaging camera (General Scanning, Planegg, Germany), was employed to detect fluorescence changes. Dual wavelength excitation at 340 and 380 nm was performed by an imaging system (Till Photonics, Planegg, Germany). After calibration, the following equation was used to relate the intensity ratios to cytosolic calcium levels: cytosolic calcium=Kd Q(R-Rmin)/(Rmax-R).

Statistical analysis

For statistical analysis unpaired Student's t-test (Stats Direct 2.2.2) was applied when appropriate. A *p*-value of less than 0.05 was considered to be significant.

Results

Influence of hypothermia on intracellular ATP concentrations

Human umbilical vein endothelial cells (HUVEC) are extremely susceptible to hypothermic injury. Nevertheless, resistance can be acquired by appropriate pre-treatment using dopamine or related compounds, as evidenced by an inhibition of LDH release. In dopamine pre-treated HUVEC, protection against hypothermic injury was transient, lasting for approximately 48hrs (fig. 1A).

To gain further insights into why dopamine pre-treated HUVEC are protected against hypothermic injury, we investigated changes in intracellular ATP levels during hypothermia. In HUVEC, 90 % of intracellular ATP was rapidly lost during the first 12hrs. Although dopamine pre-treatment resulted in a significant retardation of ATP depletion, ATP levels still decreased after 24hrs of hypothermia (fig. 1B).

Figure 1

A **B**

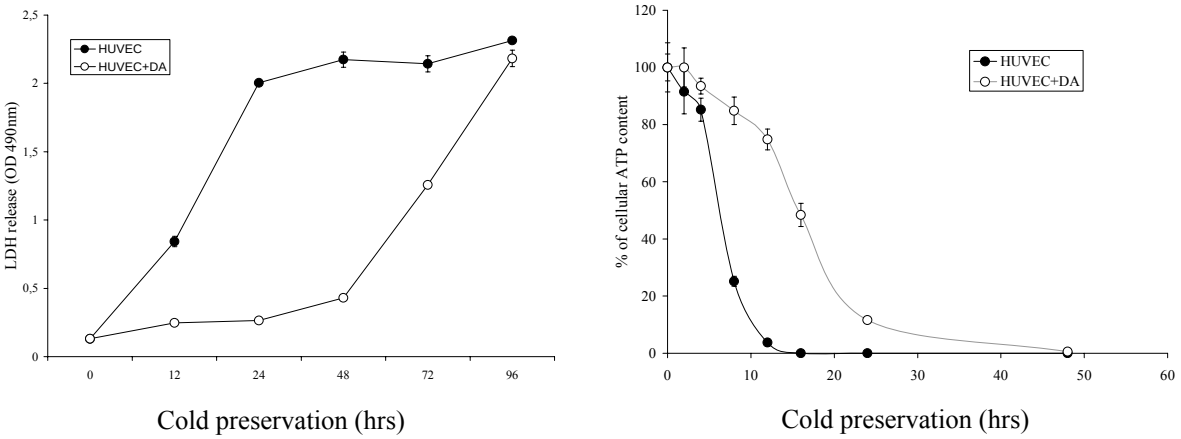


Figure 1 A: *Dopamine pre-treatment renders HUVEC transiently resistant to hypothermia induced cell death. Untreated HUVEC (black circles) and dopamine treated (25µM for 2hrs) HUVEC (open circles) were subjected to hypothermia for different time periods. Directly after cold storage cell viability was assessed by LDH release in supernatants. All experimental conditions were performed in triplicate. The results of a representative experiment are expressed as mean LDH release ± SD. A total of 4 experiments were performed.* ***B:*** *Loss of intracellular ATP during hypothermia in HUVEC. Untreated HUVEC (black circles) and dopamine pre-treated (25µM for 2hrs) HUVEC (open circles) were subjected to hypothermia as in A. ATP concentrations were assessed before and directly after various time points of hypothermia. All experimental conditions were performed in triplicate The results are expressed as mean % of ATP ± SD relative to the amount of ATP before hypothermia. A total of 5 experiments were performed.*

Influence of hypothermia on intracellular redox balance

We next assessed whether hypothermia is affecting the intracellular redox balance. At early time points after hypothermia, ROS production was not significantly different from control cells kept at 37°C. Four hours after initiation of hypothermia however, the amount of SH-reduction equivalents was significantly decreased (fig. 2A). As hypothermia continued, ROS production decreased approximately by 50 % while the amount of SH-reduction equivalents dropped to less than 10% after 24hrs (fig. 2B). Similarly, in dopamine pre-treated HUVEC, ROS production was decreased during hypothermia, but the loss of SH-reduction equivalents was significantly lower in these cells (fig. 2A and B). To assess to what extent loss of SH-reduction equivalents and ROS production affects the redox balance, we calculated the ROS/SH ratio. In untreated versus dopamine treated HUVEC, the ROS/SH ratio was 2- and 7-fold higher, after 4 and 24hrs of hypothermia respectively (fig. 2C).

Figure 2

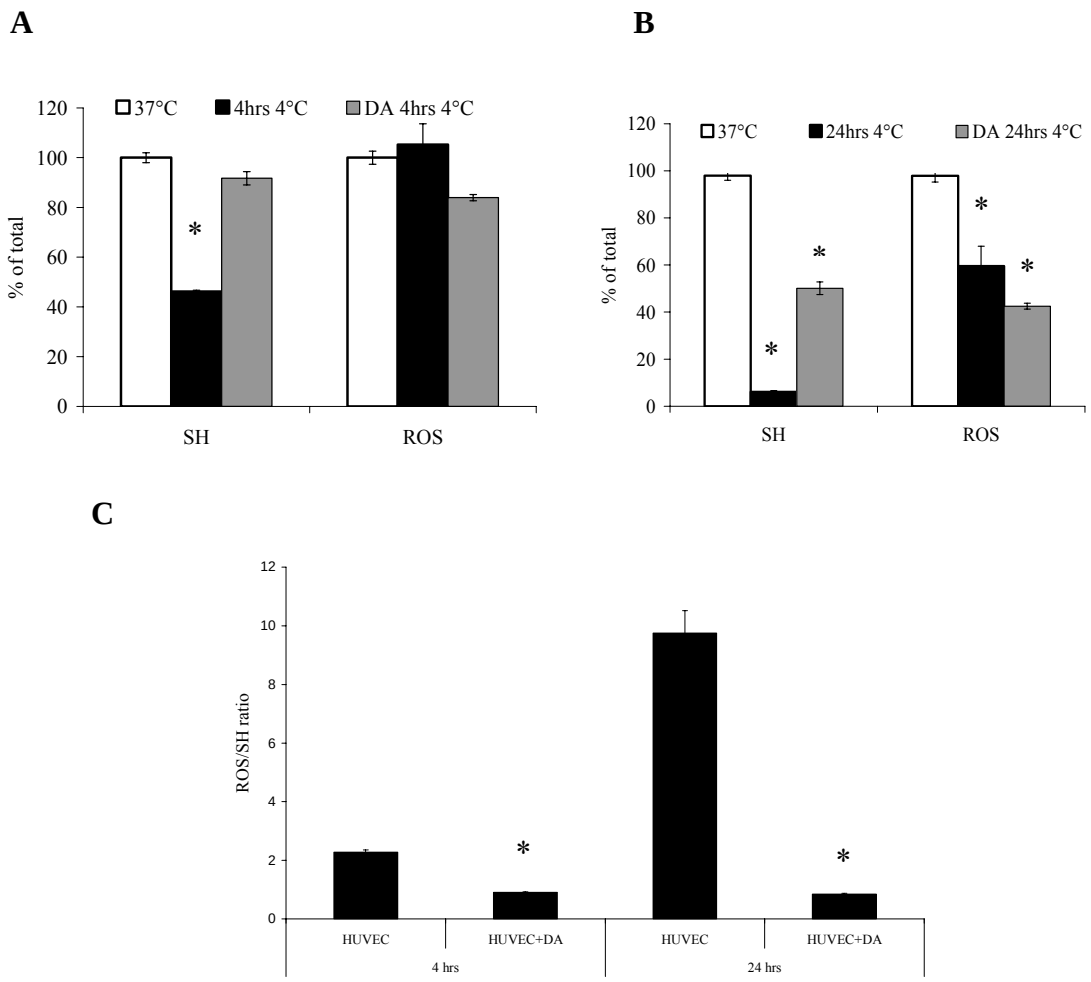


Figure 2 *Influence of hypothermia on intracellular redox balance of HUVEC. The amount of free SH-reduction equivalents and the production of ROS were assessed in untreated HUVEC (black bars) and dopamine pre-treated (25µM for 2hrs) HUVEC (grey bars) 4hrs* ***(A)*** *or*

*24hrs **(B)** after initiation of hypothermia. SH-reduction equivalents and ROS produced in HUVEC kept at 37ºC for 4hrs (in Figure 2A) or for 24hrs (in Figure 2B) (open bars) was taken as 100 %. In A and B, the results are expressed as mean % of free SH-reduction equivalents or % of ROS ± SD relative to cells kept at 37ºC. All experimental conditions were performed in triplicate, a total of 5 experiments were performed. C: The ratio of ROS and free SH-reduction equivalents after 4 and 24hrs of hypothermia. The results are expressed as mean ROS/SH ratio ± SD for all 5 experiments (*: p<0.01, unpaired t-test).*

Role of Ca^{2+} in hypothermia mediated cell death

The loss of ATP and the redox imbalance during hypothermia can affect calcium homeostasis by impairing ATP dependent ion channels. We therefore tested if a calcium influx occurs during hypothermia and if this was required for cell death. Our results showed that the addition of EDTA to the preservation solution completely prevented hypothermia mediated cell death in HUVEC (fig 3A). In time-course experiments, the addition of EDTA could

Figure 3 A **B**

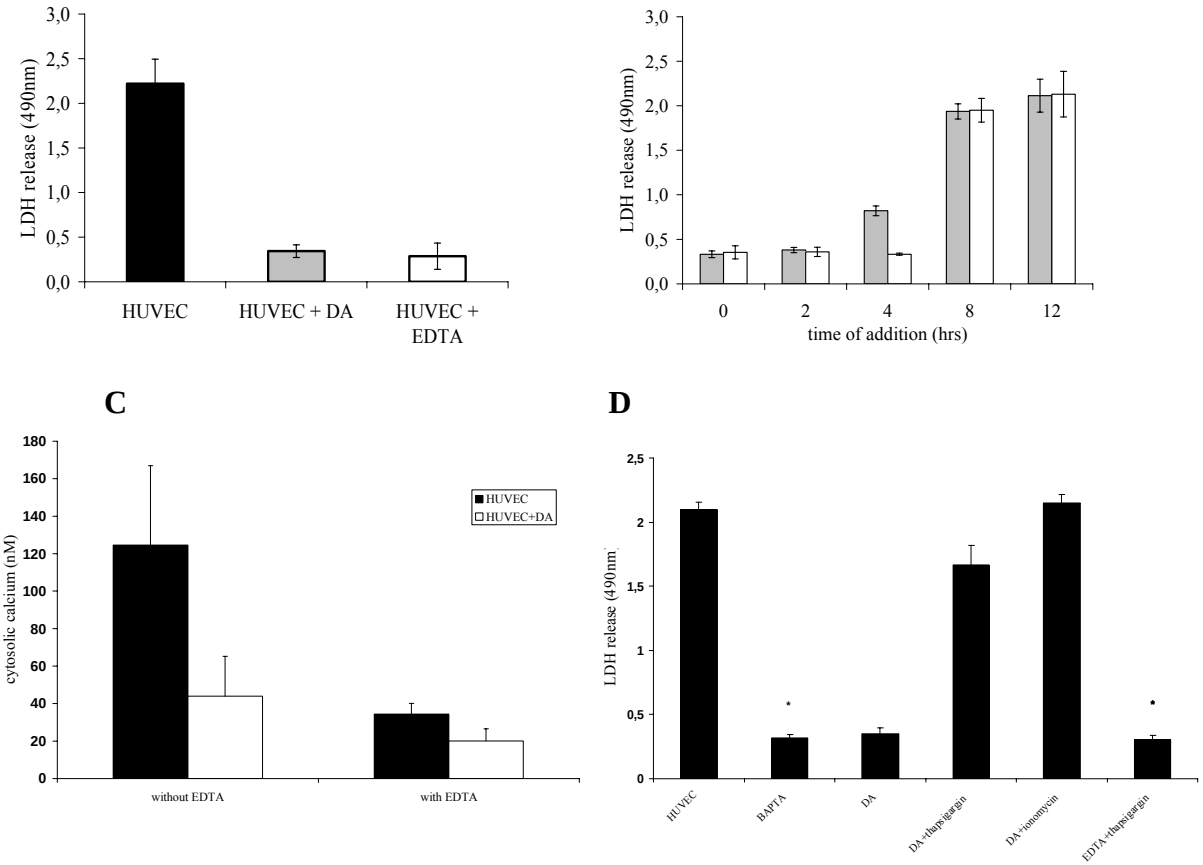


Figure 3 Role of Ca^{2+} in hypothermia mediated cell death. A: Untreated HUVEC were stored for 24hrs at 4ºC in the absence (black bar) or presence (open bar) of 10mM EDTA. In addition, DA pre-treated (25µM for 2hrs) HUVEC (grey bars) were included in the experiments as positive control. Supernatants were collected and assessed for LDH release. B: DA (25µM) (grey bars) or EDTA (10mM) (open bar) were added to HUVEC at different time

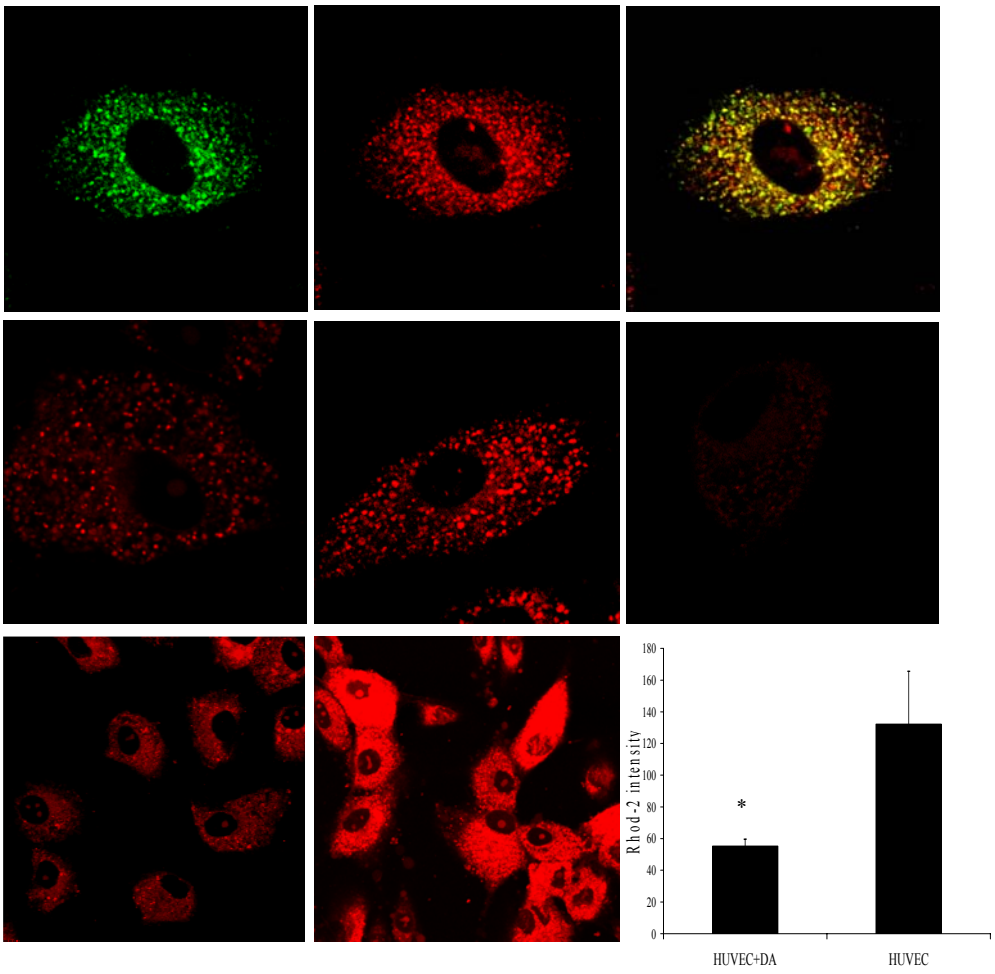
points after initiation of hypothermia. Supernatants were collected after 24hrs of hypothermia and assessed for LDH release. In A and B, results are expressed as mean LDH release ± SD. All experimental conditions were performed in triplicate, a total of 4 experiments have been performed. C: Cytosolic [Ca^{2+}] was measured in untreated (black bars) and DA pre-treated (white bars) HUVEC after 3hrs of hypothermia. Cells were subjected to hypothermia in the absence or presence of 10mM EDTA. The results are expressed as mean [Ca^{2+}] ± SD. A total of 4 experiments were performed, comprising at least 50 cells for each condition. D: HUVEC were pre-treated with 25µM DA for 2hrs and subsequently subjected to hypothermia for 24hrs in the absence or presence of thapsigargin (10µM) or ionomycin (10µM). Untreated HUVEC stored for 24hrs at 4ºC in the presence or absence of BAPTA (10µM) or in the presence of EDTA and thapsigargin (10µM) were also included in these experiments. Supernatants were collected and assessed for LDH release. The results are expressed as mean LDH release ± SD. All experimental conditions were performed in triplicate, a total of 4 experiments were done (: p<0.01, unpaired t-test).*

prevent hypothermia mediated cell death when administered for up to 4hrs after initiation of hypothermia. Beyond this time point, addition of EDTA did not protect HUVEC from cell death. Similarly, when dopamine was added at different time points after hypothermia it was not protective when the cells were already subjected to hypothermia for more than 2hrs (Fig 3B).

Intracellular [Ca^{2+}], measured after the first 3hrs of hypothermia, was significantly higher in untreated than in dopamine treated HUVEC. When EDTA was added to the preservation solution no increase in intracellular [Ca^{2+}] occurred (Fig 3C). While addition of the calcium ionophore ionomycin completely abolished the protective effect of dopamine, this was not observed in the presence of EDTA. Similarly, addition of thapsigargin to release Ca^{2+} from intracellular stores, only affected dopamine mediated protection but not that of EDTA (Fig 3D). The intracellular Ca^{2+} chelator BAPTA-AM was also protective against hypothermia mediated cell death.

To investigate if an increased Ca^{2+} influx resulted in mitochondrial Ca^{2+} accumulation, the mitochondrial calcium indicator Rhod-2 was used to assess changes in $[Ca^{2+}]_m$. The specificity of Rhod-2 for mitochondrial calcium was demonstrated by double labelling with Mitotracker green. Because entry of Rhod-2 in the mitochondria is completely dependent on the mitochondrial membrane potential, the specificity of Rhod-2 was also tested by adding CCCP to destroy the mitochondrial membrane potential. Based on the time-course experiments with EDTA, we anticipated that calcium entry would occur within the first 4hrs of hypothermia. 3hrs of hypothermia led to a strong increase in $[Ca^{2+}]_m$, which was significantly inhibited by dopamine pre-treatment (Fig. 4). Like dopamine, BAPTA-AM prevented mitochondrial Ca^{2+} accumulation. The effect of dopamine on the mitochondrial Ca^{2+} influx could be overcome by ionomycin.

Figure 4 A



B

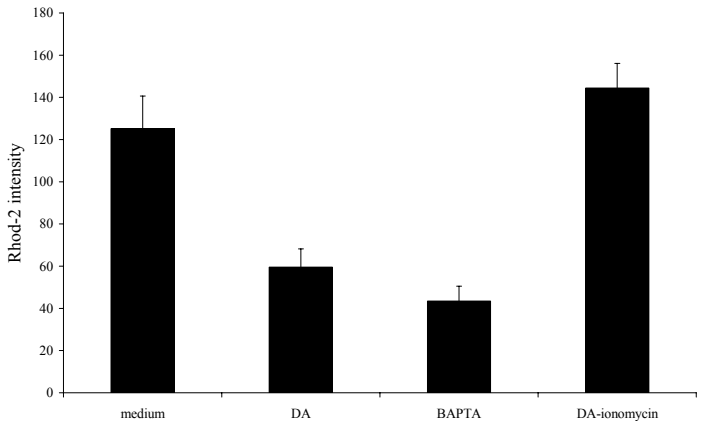


Figure 4 *Ca^{2+} is accumulated in mitochondria during hypothermia.* ***A:*** *HUVEC were incubated 2hrs prior to cold storage with 25µM DA or not. The cells were washed and labelled with Rhod-2 and Mitotracker-green directly before hypothermic preservation. 3hrs after initiation of hypothermia, the cells were analysed by confocal microscopy using the corresponding excitation and emission wave lengths. Specificity of Rhod-2 staining for mitochondrial Ca^{2+} was demonstrated by co-localization with Mitotracker-green and the*

absence of Rhod-2 staining when the cells were treated with CCCP to dissipate the mitochondrial membrane potential. ***B:*** *HUVEC were pre-treated with 25µM of DA for 2hrs and subsequently subjected to hypothermia in the absence or presence of ionomycin (10µM). Untreated HUVEC stored at 4ºC in the presence or absence of BAPTA (10µM) were also included in these experiments. HUVEC were labelled prior to cold storage as in A. Rhod-2 fluorescence was measured 3hrs after initiation of hypothermia. The results are expressed as mean Rhod-2 fluorescence intensity ± SD (*: p<0.01, unpaired t-test). A total of 4 experiments were performed, comprising at least 30 cells for each condition.*

Respiratory chain inhibitors

A consequence of mitochondrial Ca^{2+} accumulation is MPT and loss of mitochondrial membrane potential ($\Delta\Psi$). During hypothermia, dopamine prevents the loss of $\Delta\Psi$ [22]. We tested if respiratory chain inhibitors (myxothiazol, Na-azide), an inhibitor of ATP synthase (oligomycin) and an uncoupler (CCCP), all of which are known to generate mitochondrial ROS, were able to overcome the protective effect of dopamine or EDTA. Myxothiazol (data not shown) and Na-azide both abrogated the protective effect of dopamine completely (Fig 5). Oligomycin and CCCP were less effective in this regard. It must be stressed that none of these compounds influenced cell viability when tested in similar concentrations on HUVEC that were kept at 37ºC (data not shown). In the presence of EDTA, HUVEC were still protected from hypothermia mediated cell death, even when Na-azide, CCCP or oligomycin were added to the cells (Fig 5).

Figure 5

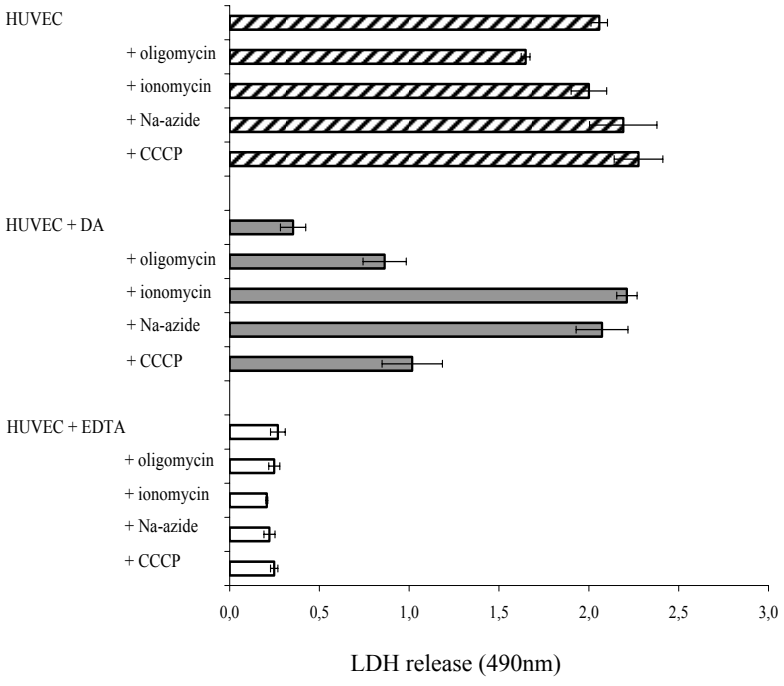


Figure 5 *Influence of respiratory chain inhibitors and ionomycin on dopamine's protective effect. Prior to cold storage, HUVEC were pre-treated with 25µM of DA for 2hrs or left*

untreated. Hereafter the cells were subjected to hypothermia for 24hrs in the absence or presence of ionomycin (10µM), oligomycin (10µM), Na-azide (10µM) or CCCP (10µM). In untreated cells hypothermia was performed in the presence or absence of 10mM of EDTA. Supernatants were collected and assessed for LDH release. The results are expressed as mean LDH release ± SD. A total of 4 experiments were performed.

Discussion

In the present paper we studied the importance of redox imbalance, changes in calcium homeostasis and depletion of ATP in hypothermia mediated cell death and evaluated the mechanisms by which dopamine protects against these deleterious effects. The main findings of this study are: first, hypothermia leads to a redox imbalance. This is not the result of an increased ROS production but rather due to depletion of SH-reduction equivalents. Second, hypothermia leads to a calcium influx, which in turn results in an increased mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$). Third, hypothermia leads to ATP depletion. Fourth, dopamine pre-treatment of HUVEC renders the cells transiently resistant to hypothermia induced cell death. The loss of SH-reduction equivalents is significantly less, ATP depletion is retarded and mitochondrial calcium accumulation is significantly inhibited under these conditions.

Recently, the role of Ca^{2+} in cold preservation damage of liver endothelial cells and hepatocytes has been questioned (27). It was suggested that cold induced injury occurred predominantly via an iron dependent pathway. Because EDTA, BAPTA-AM and even dopamine, also have the propensity to chelate iron, our results do not unequivocally point out towards a critical role for Ca^{2+} in this process. Yet, if iron chelation is the major mechanism by which dopamine exerts its protective effect, a number of issues have to be addressed. Importantly, it does not explain why dopamine prevents intracellular Ca^{2+} accumulation. Furthermore, it does not explain why Ca^{2+} release from the intracellular stores can overcome the protective effect of dopamine. The protective effect of dopamine is strictly redox dependent (22), however, we can not exclude that oxidation of dopamine impairs the ability to chelate iron. Unpublished findings also have shown that protection mediated by dihydroxy phenolic compounds, was only observed when the hydroxyl groups were in ortho- or para- but not in meta-position. While any of these compounds are able to chelate iron, the meta-dihydroxy phenolic compounds cannot be oxidized, and hence cannot donate reduction equivalent. This also emphasizes that iron chelation is unlikely underlying the protective effect of dopamine.

There is convincing evidence that redox potential can influence Ca^{2+} release from the endoplasmatic reticulum (ER) (28). Koshita et al (29) found that Ca^{2+} release from the

sarcoplamatic reticulum could be induced by oxidizing compounds and that this was blocked in the presence of GSH. In untreated HUVEC depletion of SH-reduction equivalents occurred already within 4 hrs of hypothermic preservation. Although the mechanism by which this occurred, i.e. oxidation or release from the cell, has not been addressed in this study, it is an eligible condition that could lead to Ca^{2+} release from the ER. Subsequently, Ca^{2+} might enter the cells via store-operated channel, leading to a critical Ca^{2+} concentration in the cytosol (30-32). A number of observations are in line with this sequel of events. Firstly, at early time-point dopamine completely prevented the depletion of SH-reduction equivalents. Although at 24 hrs also in dopamine treated cells SH-reduction equivalents were partially depleted, this was significant less compared to untreated cells. Secondly the protective effect of dopamine could be abrogated by thapsigargin, a compound that prevents the re-uptake of Ca^{2+} in the ER and hence results in partial depletion of Ca^{2+} from the ER through leakage into the cytosol. Because thapsigargin could not abrogate cell protection when cold preservation was performed in the presence of EDTA, this indicates that the amount of Ca^{2+} released from the ER is not sufficient for cell death and that cell death dependents on additional Ca^{2+} influx.

This study does not argue against an important role for iron in cold preservation injury. In fact, a number of studies already have shown the importance of iron in this respect. Kerkweg et al. (23) have demonstrated that the chelatable iron-pool is increased during hypothermia. Moreover, studies using deferoxamine have clearly demonstrated the beneficial effect of iron chelating on hypothermia mediated cell death (23, 24). Over-expression of HO-1 has also been shown to be beneficial with respect to hypothermic preservation injury (33). This is partly mediated via the generation of CO as reviewed by Nakao et al. (34). CO can bind iron directly and hence might prevent the formation of hydroxyl radicals by Fenton chemistry. Formation of radicals in conjunction with depletion of SH-reduction equivalents will result in a redox imbalance, thereby linking the iron dependent pathway with Ca^{2+} overload.

Since ATP is required to maintain Ca^{2+} homeostasis, it can be argued that suppression of mitochondrial activity under hypothermic conditions might be the initial event leading to Ca^{2+} overload. Based on time course experiments however, an increased $[Ca^{2+}]_m$ was evident within 3hrs of hypothermia, while ATP depletion was marginally influenced at this time-point. Although in dopamine treated cells ATP depletion was also observed after 24 hrs of hypothermic preservation, we have previously demonstrated that at this time-point the mitochondrial membrane potential ($\Delta\Psi$) was still intact (22). Inasmuch as respiratory chain inhibitors or uncouplers could overcome the protective effect of dopamine, this was unlikely due to an impaired ATP generation as hypothermia *per se* is associated with a decreased

mitochondrial activity. Nevertheless, these compounds are able to generate mitochondrial ROS production (33), which subsequently might leak to the cytosol, oxidize dopamine and thus abolish its protective effect.

Based on our results and data from the literature we propose the following model of hypothermic preservation injury as depicted in Figure 6. We are aware that this model should not be generalized for all cells or tissues. Nevertheless, it explains why the protective effect of dopamine is redox dependent and why dopamine prevents Ca^{2+} overload during hypothermic preservation.

Figure 6

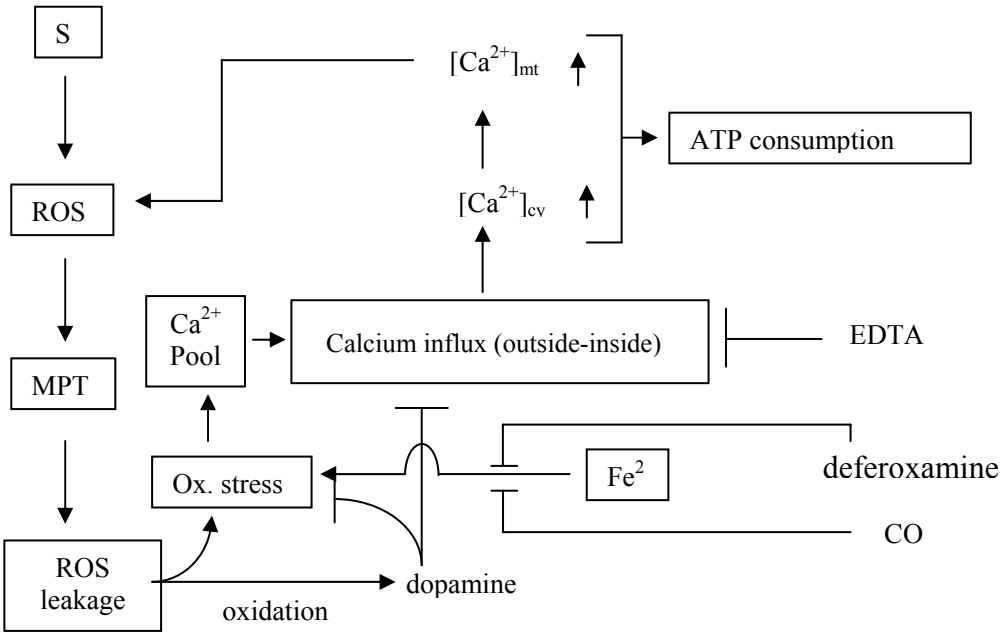


Figure 6 *Proposed model for hypothermia mediated damage and mechanism by which protection can be achieved.*

What are the possible implications from this study for organ preservation? One important implication is that during organ preservation cellular calcium entry must be prevented. To avoid the deleterious effects of Ca^{2+} accumulation in the cells, Ca^{2+} has been omitted from most preservation solutions (35). However, these solutions are not completely devoid of Ca^{2+} since a small amount of this cation is likely to be present in the solvent. Moreover, complete omission of Ca^{2+} can lead to opening of unselective cation channels and hence to membrane depolarization. In fact, addition of small amounts of calcium to preservation solutions has proven to be more protective in experimental liver transplantation (36, 37). Based on the

proposed model, prevention of cellular calcium entry can be achieved either by reducing iron mediated oxidative stress or by preventing the occurrence of a redox imbalance. In the present study we have demonstrated that dopamine is capable to prevent redox imbalance. Because this phenomenon is not exclusive for dopamine, but holds true for other hydrophobic dihydroxyphenolic compounds (22), the use of such compounds devoid of hemodynamic action are of potential clinical relevance to prevent hypothermic preservation of organ allografts.

References

1. Muller FL, Roberts AG, Bowman MK, Kramer DM. Architecture of the Qo site of the cytochrome bc1 complex probed by superoxide production. Biochemistry 2003; 42: 6493-6499
2. St-Pierre J, Lin J, Krauss S, Tarr PT, Yang R, Newgard CB, Spiegelman BM. Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. J Biol Chem 2003; 278: 26597-26603
3. Brand MD, Buckingham JA, Esteves TC et al. Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. Biochem Soc Symp 2004; 71: 203-213
4. Komatsu M, Kuroda M, Wang Y et al. Manganese superoxide dismutase overexpression changes plating efficiency bidirectionally according to change in redox for SaOS2 human osteosarcoma cell line. Int J Oncol 2005; 26: 853-862
5. Gunter TE, Gunter KK, Sheu SS, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. Am J Physiol 1994; 267: C313-339
6. Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD. Calcium and mitochondria. FEBS Lett 2004; 567: 96-102
7. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 2004; 287: C817-833
8. Balaban RS. Cardiac energy metabolism homeostasis: role of cytosolic calcium. J Mol Cell Cardiol 2002; 34: 1259-1271
9. Hansford RG, Zorov D. Role of mitochondrial calcium transport in the control of substrate oxidation. Mol Cell Biochem 1998; 184: 359-369
10. Mildaziene V, Baniene R, Nauciene Z et al. Calcium indirectly increases the control exerted by the adenine nucleotide translocator over 2-oxoglutarate oxidation in rat heart mitochondria. Arch Biochem Biophys 1995; 324: 130-134
11. Aon MA, Cortassa S, Akar FG, O'Rourke B. Mitochondrial criticality: a new concept at the turning point of life or death. Biochem Biophys Acta 2006; 1762: 232-240
12. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 2000; 192: 1001-1014
13. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int 2004; 65: 713-718
14. Rauen U, de Groot H. New insights into the cellular and molecular mechanisms of cold storage injury. J Investig Med 2004; 52: 299-309
15. Boutilier RG. Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol 2001; 204: 3171-3181
16. Muleme HM, Walpole AC, Staples JF. Mitochondrial metabolism in hibernation: metabolic suppression, temperature effects, and substrate preferences. Physiol Biochem Zool 2006; 79: 474-483
17. Boyer BB, Barnes BM, Lowell BB, Grujic,D. Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels. Am J Physiol 1998; 275: R1232-1238
18. Liu B, Belke DD, Wang LC: Ca2+ uptake by cardiac sarcoplasmic reticulum at low temperature in rat and ground squirrel. Am J Physiol 1997; 272: R1121-1127
19. Wang SQ, Lakatta EG, Cheng H, Zhou ZQ. Adaptive mechanisms of intracellular calcium homeostasis in mammalian hibernators. J Exp Biol 2002; 205: 2957-2962
20. Ahlenstiel T, Burkhardt G, Kohler H, Kuhlmann MK. Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. Kidney Int 2003; 63: 554-563
21. Ahlenstiel T, Burkhardt G, Kohler H, Kuhlmann MK. Improved cold preservation of kidney tubular cells by means of adding bioflavonoids to organ preservation solutions. Transplantation 2006; 81: 231-239
22. Yard B, Beck G, Schnuelle P et al. Prevention of Cold-Preservation Injury of Cultured Endothelial Cells by Catecholamines and Related Compounds. Am J Transplant 2004; 4: 22-30
23. Kerkweg U, Li T, de Groot H, Rauen U. Cold-induced apoptosis of rat liver cells in University of Wisconsin solution: the central role of chelatable iron. Hepatology 2002; 35: 560-567
24. Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 2004; 287: F181-187
25. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52:2745-56
26. Koppel H, Yard BA, Christ M, Wehling M, van der Woude FJ. Modulation of angiotensin II-mediated signalling by heparan sulphate glycosaminoglycans. Nephrol Dial Transplant 2003; 18: 2240-2247
27. Knoop S, de Groot H, Rauen U. Little evidence for a major role of Ca2+ in cold-induced injury of liver cells. Cryobiology 2008; 56: 103-13

28. Pessah IN. Ryanodine receptor acts as a sensor for redox stress. Pest Manag Sci 2001; 57: 941-5
29. Koshita M, Miwa K, Oba T. Sulfhydryl oxidation induces calcium release from fragmented sarcoplasmic reticulum even in the presence of glutathione. Experientia. 1993; 49(4):282-4.
30. Groschner K, Hingel S, Lintschinger B et al. Trp proteins form store-operated cation channels in human vascular endothelial cells. FEBS Lett. 1998; 437(1-2):101-6.
31. Harder DR. Ca-induced Ca release: lessons regarding cell models. Am J Physiol Heart Circ Physiol 2004; 287: H1885-1886
32. Sparagna GC, Gunter KK, Sheu SS, Gunter TE. Mitochondrial calcium uptake from physiological-type pulses of calcium, a description of the rapid uptake mode. J Biol Chem 1995; 270: 27510-27515
33. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. Biochim Biophys Acta 2006; 1757: 509-517
34. Nakao A, Kimizuka K, Stolz DB et al. Protective effect of carbon monoxide inhalation for cold-preserved small intestinal grafts. Surgery 2003; 134: 285-292
35. Lang F, Busch GL, Gulbins E. Physiology of cell survival and cell death: implications for organ conservation. Nephrol Dial Transplant 1995; 10: 1551-1555
36. Ben Abdennebi H, Steghens JP, Hadj-Aissa A et al. A preservation solution with polyethylene glycol and calcium: a possible multiorgan liquid. Transpl Int 2002; 15: 348-354
37. Dutkowski P, Wahl W, Winkelbach V, Watzka M, Krysiak M, Junginger T. Calcium prevents loss of glutathione and reduces oxidative stress upon reperfusion in the perfused liver. *Int J Surg Investig* 2000; 2: 1-7

Chapter 4

Protection against cold preservation injury by catecholamines is linked to redox activity and lipophilicity

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*: contributed equally to the work.

Manuscript submitted

Abstract

Previous studies have demonstrated that catecholamines protect endothelial cells against cold preservation-induced damage. Based on the differences in potency of protection amongst catecholamines and related substances, we hypothesized that variation of the molecular structure may yield more efficacious compounds.

Modification of dopamine by an alkanoyl group greatly improved efficacy of protection. This was associated with increased cellular uptake and loss of hemodynamic action. Further variation revealed that only compounds bearing two hydroxyl groups in ortho- or para-position at the benzene nucleus, i.e. strong reductants, were protective and the protective efficiency were correlated with logP value. Although a strong reducing capacity was required for protection, general antioxidants, e.g. N-acetyl cysteine and ascorbate, did not prevent cold preservation-induced cell damage. Similarly, inhibition of NADPH oxidase had no effect on cell viability during hypothermia. Although dopamine increases heme oxygenase 1 (HO-1) expression, its protective effect is not mediated by this enzyme as shown by knockout experiments. In conclusion, our data demonstrate that protection against cold preservation injury by catecholamines is exclusively mediated by two structural entities, i.e. strong reducing capacity and sufficient lipophilicity.

Introduction

Hypothermic preservation of allografts is common practice in transplantation medicine. However, organ quality deteriorates with increasing cold ischemia time. Thus, hypothermic preservation is also a major cause of pre-transplantation injury (1, 2). The mechanism behind prolonged hypothermic preservation damage has been the subject of many studies over the past decades. Ultimately, these studies have resulted in major improvements of preservation techniques and cold storage solutions. Already in the early days of organ preservation, it became evident that hypothermic preservation leads to ATP depletion, dysregulation in calcium homeostasis and eventually cell swelling (3). The use of hyperosmolaric solutions has been shown to limit the problem of cell swelling, however, the problems that are associated with ATP depletion during hypothermic preservation of solid organs are not solved yet.

The ATP-dependent endothelial barrier function, which prevents paracellular transport of proteins and solutes, is severely compromised during hypothermic preservation. This is due to the rearrangement of endothelial adherence and tight junction proteins as well as to changes in F-actin, vimentin and α-tubulin expression (4). The loss of endothelial barrier function might explain the formation of tissue oedema in allografts that have been subjected to long periods of hypothermic preservation.

In hibernating mammals body temperature can decrease as low as 4°C without causing any apparent harm to these animals. Even when body temperature is increasing during short periods of arousal, no tissue damage occurs. This tolerance against hypothermia is an adaptive response, which, from an evolutionary point of view, has been lost in men. Catecholamines play a pivotal role in the hibernation response as they inhibit apoptosis of brown adipose tissue in a beta-receptor dependent fashion (5). Interestingly, we have demonstrated that catecholamines have the propensity to protect human endothelial cells against cold preserevation-induced damage (6, 7). This protection is not receptor mediated, does not require *de novo* protein synthesis and is completely abolished by prior oxidation of catecholamines (8). It is also clear from other studies that several catechol-containing compounds, e.g. bioflavonoids or other structures that are readily oxidized to form (homo-) quinones (9), behave similar to catecholamines with regard to their cryoprotective effect. Therefore, we believe that structural entities within these compounds might be collectively responsible for protection. Amongst these entities, the catechol moiety is of particular interest. Catechols, i.e. ortho-dihydroxyphenols, can be readily oxidized and therefore can act as

scavenger for reactive oxygen species (ROS) (10). Moreover, catechols are of critical importance for iron acquisition in prokaryotes, by virtue of their ability to bind iron (10, 11). As has been shown for deferoxamin, iron chelation protects cells against hypothermia-induced injury (12).

As implicated by their nomenclature, all catecholamines contain the catechol structure. Yet, they differ extremely in their efficacy to protect endothelial cells against hypothermic preservation injury (8). This suggests that apart from the catechol moiety, also the amine side chain must play an important role in protection.

We hypothesized that modification of the catecholamine structure will yield more efficacious compound. In the present study we investigated the molecular entities within catecholamines that convey protection, by systematic variation of their molecular structure. This included alterations in their lipophilicity, by attachment of various alkyl- or aryl-derived residues in the amine side chain and alterations in their reducing capacity by modifications of the substituents at the benzene nucleus. As a consequence, these newly synthesized molecules strongly differ in logP values and reducing capacities, thus enabling to dissect the structural requirements for catecholamines that convey protection.

Materials and Methods

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously (25). The cells were cultured in endothelial cell growth medium (EGM) (Promocell, Heidelberg, Germany) in T25 or T75 flasks (Greiner, Frickenhausen, Germany) coated with gelatine (1%). Confluent monolayers were passaged by Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO). Characterization of endothelial cells was performed on the basis of a positive uptake of acetylated LDL, Factor VIII related antigen and PECAM (CD31) expression, and negative staining for smooth muscle alpha actin.

Hypothermic preservation injury

Hypothermic preservation injury of HUVEC was assessed by lactate dehydrogenase (LDH) release. LDH assays were performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, HUVEC were plated in 24-well plates, grown until confluence and pre-incubated with test substances for 2 hrs. The plates were washed three times with 1ml of PBS and stored for 24 hrs at 4°C in phenol red free medium. A 100µl

aliquot of each supernatant was used to determine LDH release. In each experiment 100µl of phenol red free medium was used as blank. The results are expressed as OD490nm corrected for the blank.

Synthesis of compounds

Test compounds were synthesized from commercially available precursors (Fluka, Neu-Ulm, Germany, unless stated otherwise). Solvents were from Merck (Darmstadt, Germany). All compounds were purified to homogeneity by recrystallization from aqueous methanol as demonstrated by thin layer chromatography (TLC) (Si 60, detection by fluorescence quenching and by charring with 5% sulfuric acid). Samples investigated by NMR (Bruker AC250) yielded spectra in accordance with the expected structures. LogP values were calculated using the engine at www.daylight.com/daycgi/clogp. All compounds synthesized in this study are shown in Table 1.

General procedure for acylated dopamine derivatives

Carboxylic acids were converted to their mixed anhydride derivatives by reacting with ethyl chloroformate, which was reacted with the appropriate amines. Briefly, 2 mmole carboxylic acid was dissolved in 10 mL acetonitrile. To this solution 2.1 mmole N-ethyldiisopropylamine was added under stirring, followed by addition of 2.1 mmole of ethyl chloroformate. Two hours later, the mixture was diluted with 15 mL ethyl acetate and washed twice with 5 mL brine. The organic layer was dried over $MgSO_4$ and the solution was used for further experiments. Dopamine hydrochloride was dissolved in DMF at 50 mg/mL, stoichometric amounts of N-ethyldiisopropylamine and ethoxycarbonylcarboxylate were added and the mixture incubated overnight protected from light. The mixture was partitioned in 1 ml 0.1 M $NaHCO_3$ and 2 mL ethyl acetate, the organic layer washed with 0.1 M H_2SO_4 and brine, and finally dried over $MgSO_4$. Evaporation of the solvent yielded the acylated product which was homogenous in TLC. Aminophenols required a five-fold excess of phenol over fatty acid as higher acylation products are easily formed (26).

General procedure for dihydroxybenzoyl amides

2.5 mmole (377 mg) of the acid was dissolved in 4 ml THF. Subsequently, 2.5 mmole amine and 2.5 mmole (515 mg) DCC, dissolved in 2 ml THF, were added to the solution and mixed. The solution was kept over night at room temperature and filtrated hereafter to remove precipitates. The filtrate was concentrated in vacuo and re-dissolved in 10 ml ethyl acetate.

The organic phase was washed in three sequential steps with 10 ml 0.5 M H_2SO_4, brine and 5% $NaHCO_3$. Finally the organic phase was washed with brine and dried over $MgSO_4$. The solvent was removed in vacuo.

[^{3}H]N-Octanoyl dopamine (5)

To 1.85 MBq 7,8-^{3}H dopamine (Amersham) dissolved in 50 µL of 0.01M acetic acid containing 50% ethanol was added, under nitrogen atmosphere, 20 µL of a 1 M solution of octanoyl-ethoxycarbonyl anhydride in ethylacetate, 10 µL pyridine and 20 µL DMF. After one hour, the reaction was mixed with 1 ml 0.1 M phosphate pH 7.5 and 0.5 mL ethyl acetate. The aqueous phase was re-extracted and the combined extracts were washed with 1 mL 0.1 M phosphate pH 3.7. About 65% of radioactivity was recovered. TLC (Si60, ethyl acetate / acetic acid 10:1), spraying with En^{3}hance (NEN) and 7 d exposure on X-ray film revealed >90% purity as judged by comigration of nonradioactive N-octanoyl-dopamine.

Tracer studies and subcellular fractionation

HUVEC cultured to confluence in T175 flasks were incubated with 925 kBq (10 µCi) of ^{3}H-N-octanoyl-dopamine or ^{3}H-dopamine for 2 hrs. Hereafter the cells were washed and processed further for subcellular fractionation. Subcellular fractionation was performed using a commercially available kit from Geno Technology Inc. (St. Louis, MO). All steps were performed at 4°C. Briefly, cell lysates were prepared and homogenized by douncing. The cell homogenate was centrifuged for 2 min at 2,000 x g. Thereafter, the supernatant containing cytosolic proteins as well as cellular organelles was further centrifuged for 2 min at 4,000 x g. Cytosolic proteins and cellular organelles were present in the supernatant (S1). The pellet (P1) contained cell membranes and the nuclear fraction. S1 was further centrifuged for 20 min at 10,000 x g to separate the cellular organelles (pellet P2) from cytosolic proteins (supernatant S2). P2 was subjected to a hypotonic shock (27) for 10 min followed by centrifugation (20min 10,000 x g) to separate mitochondria (pellet P3) from lysosomes (supernatant S3). Supernatant S2 was further centrifuged for 1h at 100,000 x g. Purity of the subcellular fractions was tested by Western blot analysis using antibodies against mitochondrial COX IV (New England Biolabs, Frankfurt, Germany) and cytosolic GAPDH (Abcam plc, Cambridge, UK). Radioactivity was measured in small aliquots collected directly after douncing and in aliquots of P1, P3 and S2. Aliquots of the purified fractions were mixed with 3 mL scintillation cocktail and counted in a Beckman LS6000.

Measurement of hemodynamic parameters

Male Fisher rats (250 g) were anaesthetised with ketamine and xylazine. Dopamine or N-octanoyl-dopamine (0.05 μmoles/kg body weight for both) was infused into the femoral vein. Systemic blood pressure (mean arterial pressure, mmHg) was continuously measured by a femoral arterial catheter (Portex Fine Bore Polythene Tubing 0,4mm ID, SIMS Portex, England).

Silencing of heme oxygenase 1 (HO-1)

HUVEC were seeded in 6-well plates (2 x 10^5 cells per well) and transfected with HO-1 siRNA (Santa Cruz, Heidelberg, Germany) according to the manufacturer's recommendation. After 24hrs the transfection medium was replaced with fresh normal growth medium for 48hrs. Thereafter, the expression of HO-1 protein was determined by Western blot analysis as described (18). Nonspecific siRNA (Santa Cruz, Heidelberg, Germany) was used as a negative control.

Results

New compounds were synthesized as described in methods and tested along with other substances for their protective effect against hypothermic damage. From the dose-response curves, EC50 values were calculated for each of the synthesized substance summarized in Table 1. Modification of dopamine by a middle- or long-chain alkanoyl group (compound 5-8) greatly improved efficacy (about 40 times compared to dopamine). The two aromatic residues employed, tosyl (compound 9) and phenylpropionyl (compound 10), also significantly improved efficacy. Although the latter two compounds were structurally related to dobutamine, they were slightly less effective than dobutamine (compound 4). Removal of the 3-hydroxyl group from the highly active lipophilic compound 5 resulted in the generation of the closely related tyramine derivative (compound 11). The efficacy of compound 11 was strongly decreased compared to compound 5. This could indicate either the requirement for a strongly reducing catechol function, or suggest a receptor mediated phenomenon requiring the intact dopamine fragment.

Table 1

Compound #	Fig	EC50 [µM]	strong reducing agent ?	logP (calc.)
1	1A	~ 75	+	0.169*
2	1A	>100	+	-0.989*
3	1A	>100	+	-0.685*
4	1A	5	+	2.433*
5	1B	2.1 ± 0.2	+	2.929
6	1B	0.9 ± 0.2	+	3.987
7	1B	1.2 ± 0.2	+	5.045
8	1B	1.3 ± 0.2	+	6.103
9	1B	12 ± 1	+	2.253
10	1B	9 ± 1	+	1.852
11	1B	>100	-	3.526
12	2	>100	+	1.622
13	2	>100	+	1.062
14	2	>100	+	1.622
15	2	>100	-	1.622
16	2	>100	-	0.992
17	2	>100	-	2.252
18	2	1.2 ± 0.1	+	4.621
19	2	2.4 ± 0.2	+	3.741
20	2	6 ± 1	+	4.6
21	2	> 100	-	4.621
22	2	> 100	-	3.671
23	2	~ 90	-	3.741

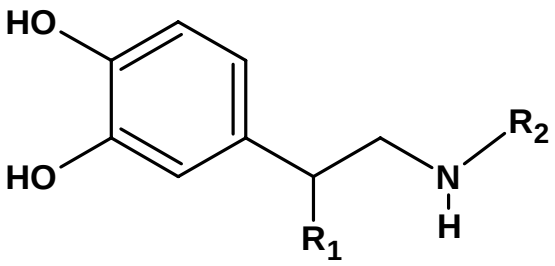
*The protective effect of various compounds against cold preservation-induced endothelial cell damage, expressed as the concentration required to inhibit 50% of LDH release, vs. lipophilicity, expressed as logP (calculated, * denotes experimental values taken from public sources).*

To test this hypothesis, we synthesized the octylamide derivatives (compound 18-23) of all possible dihydroxybenzoic acids (compound 12-17), comprising three reducing and three

non-reducing (or weakly-reducing) structures. While the free acids (compound 12-17) were all ineffective, the reducing octylamides (compound 18 – 20) exhibited EC50 values between 1.2 and 6 μM. In addition, the non-reducing octylamides (compound 21 – 23) that cover the same range of lipophilicity were all ineffective. In addition, we tested the hemodynamic action of dopamine against that of compound 5. As expected from the loss of charge on nitrogen in the octanoyl derivative 5, it did not influence mean arterial pressure *in vivo*, compatible with the hypothesis that n-acylation of dopamine impairs receptor binding.

Figure 1 Structures of compounds used in this study.

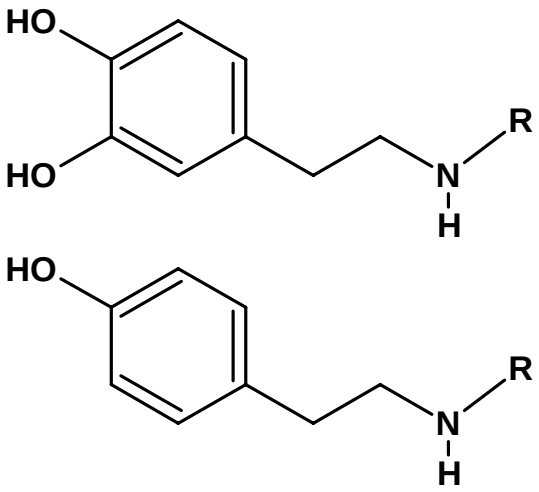
A.



R_1	R_2	
H	H	**1** Dopamine
OH	H	**2** Noradrenaline
OH	CH_3	**3** Epinephrine (Adrenaline)
H	4-(4-hydroxyphenyl)butyl(2)	**4** Dobutamine

***A**: Catecholamines previously reported to exert protective effect on endothelial cells against cold preservation-induced damage.*

B.

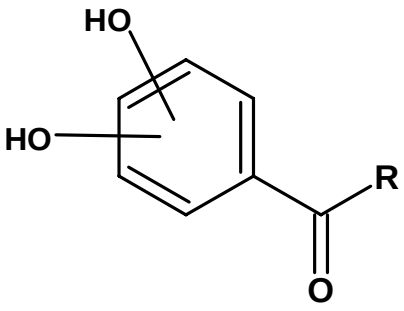


R	
n-octanoyl	**5**
n-decanoyl	**6**
n-dodecanoyl	**7**
n-tetradecanoyl	**8**
p-tosyl	**9**
phenylpropionyl	**10**

R = n-octanoyl **11**

***B**: Structurally related derivatives of dopamine and a derivative lacking one of the two hydroxyl moieties at the aromatic ring.*

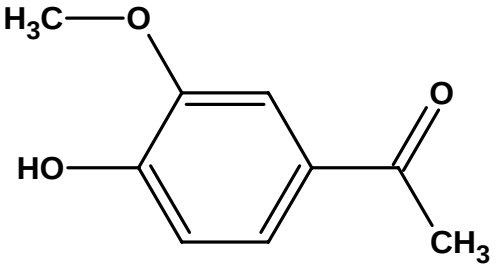
C.



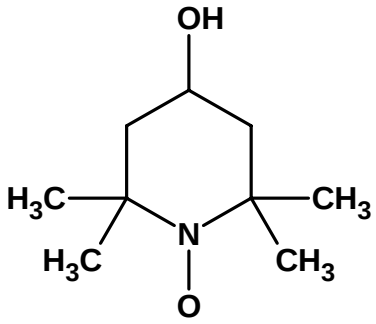
position of OH moieties	R=OH	R=NH-C_8H_{17}
2,3	**12**	**18**
3,4	**13**	**19**
2,5	**14**	**20**
2,4	**15**	**21**
3,5	**16**	**22**
2,6	**17**	**23**

***C**: Chemically possible isomers of the dihydroxybenzoyl fragment used as free acids (R = OH) or as much more lipophilic n-octyl amides (R = C_8H_{17}). While the compounds with hydroxyl groups in ortho- or par-position (2,3; 3,4 or 2,5) are strong reducing agents, the other isomers are much weaker.*

D.



Apocynin **24**



Tempol **25**

***D**: Structure of the chemically unrelated substances apocynin (a NADPH oxidase inhibitor) and tempol (2,2,6,6-tetramethylpiperidine-1-oxyl), a stable radical often employed as a low molecular weight superoxide dismutase mimetic.*

All compounds identified as protective cover diverse structures with only one common property, i.e. the reducing capacity. Therefore, it is unlikely that a specific receptor interaction is involved in the protective properties of these compounds. In order to investigate whether the protection reflects a general antioxidant effect, we also employed other biologically relevant reducing compounds and specific inhibitors of certain oxidative pathways. Both ascorbate and N-acetyl cysteine were not protective when used up to 300 µM. Similarly, apocynin (24), an inhibitor of NADPH oxidase, did not protect HUVEC from cold preservation induced damage at concentrations up to 3 mM. The stable radical tempol

(2,2,6,6-tetramethylpiperidin-n-oxyl) (25), a scavenger of reactive oxgen species, was also not protective at concentrations up to 300 µM .

Collectively our experimental data thus suggest that a highly protective agent requires a suitably substituted dihydroxybenzene moiety and a certain degree of lipophilicity. The relationship between relative hydrophobicity (logP) and protection (EC50) is given in figure 2. The maximal protective effect (EC50 at 1-2 µM) requires logP to be approximately 3 or higher.

Figure 2

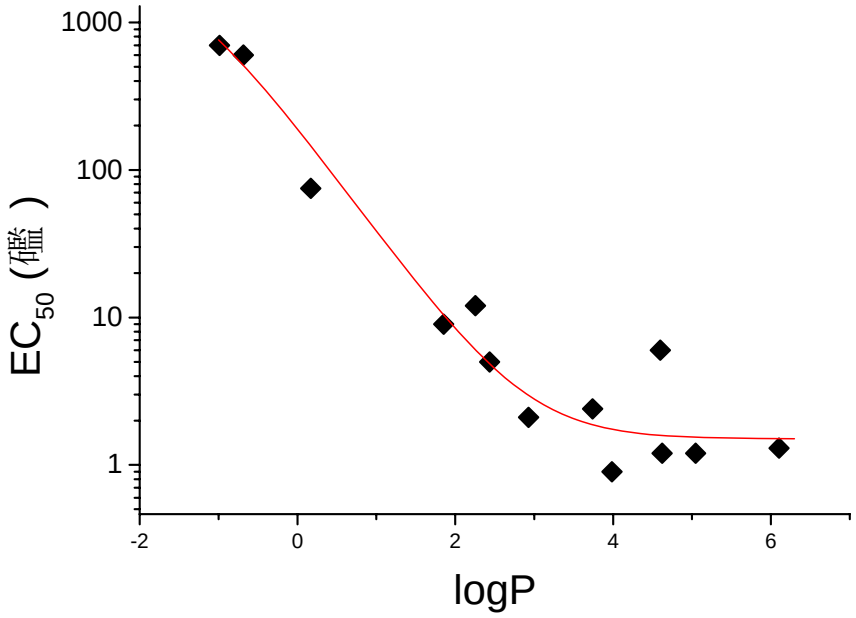


Figure 2 *EC50 values expressed as mean plotted against calculated logP values for the reducing compounds shown in table 1. A sigmoid (Boltzmann) line was found to fit the data set with A1= 1.22, A2=1000, x0=-0.67, dx = -0.58*

To investigate how the change in lipophilicity affects the cellular uptake of the compounds and their distribution in different subcellullar compartments, [^{3}H] labeled compound 5 was synthesized from 7,8-[^{3}H] dopamine. Based on the amount of radioactive [^{3}H] label, total uptake of compound 5 was approximately 4 times higher than the unmodified dopamine in HUVEC. Subcellular distribution of these compounds remained almost unaffected, with the exception of mitochondria. The relative concentration of compound 5 was about twice as much compared to dopamine, corresponding to approximately 8 times more radioactivity in the mitochondria when compound 5 was tested against dopamine.

Since HO-1 over-expression has been shown to be beneficial with respect to hypothermia-mediated cell damage (13), we investigated the influence of hypothermia on HO-1 expression and how this was modulated in dopamine pre-treated HUVEC. Dopamine did not significantly change the expression of HO-1 directly before cold storage. However, when HUVEC were stored at 4°C HO-1 expression was significantly diminished in untreated HUVEC. In contrast, in dopamine pre-treated cells HO-1 expression was maintained during hypothermia (Figure 3A). This could indicate that the protective effect of dopamine is mediated by stabilization of HO-1. To test this hypothesis, knockdown experiments using HO-1 siRNA were performed. HO-1 expression was almost completely lost 3 days after siRNA transfection. Even 5 days after transfection HO-1 expression did not reappear in transfected cells, and this was not influenced by addition of dopamine on day 4 (Figure 3B). HO-1 siRNA transfection could not overcome the protective effect of dopamine (Figure 3C). Hence, protection against hypothermia-mediated cell death by catechol compounds does not require HO-1 expression.

Figure 3

A

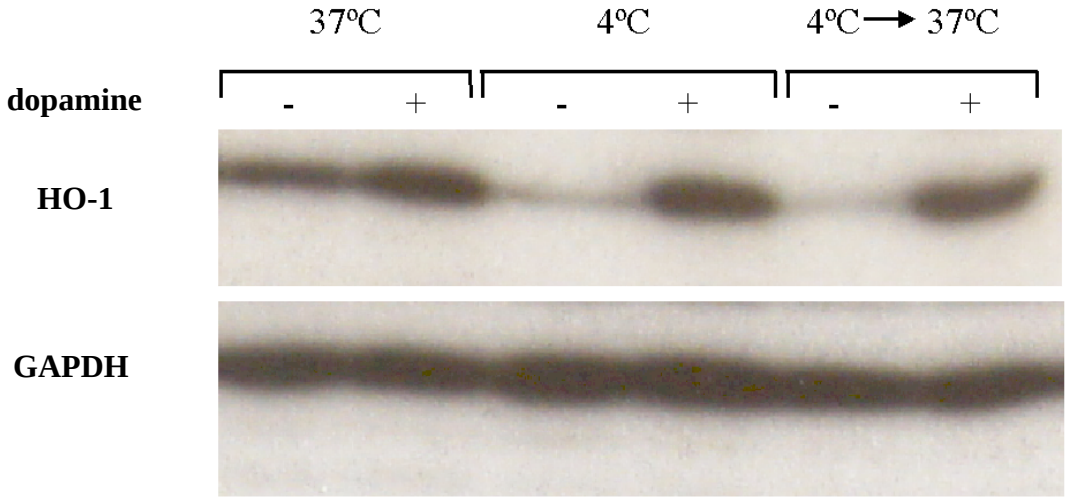
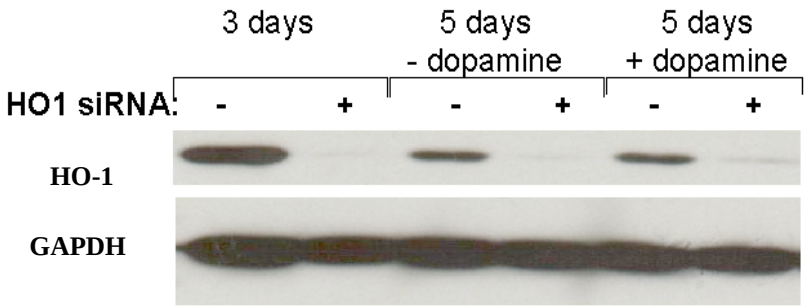


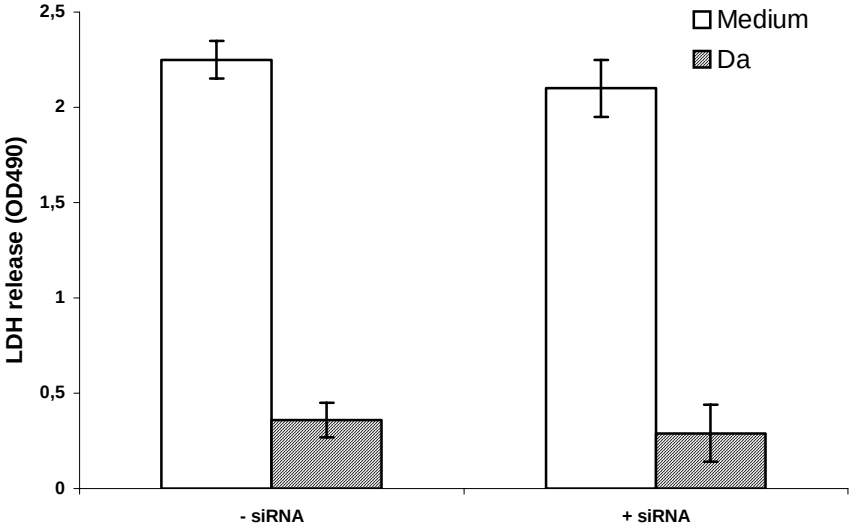
***Figure 3 A**: Influence of hypothermia on HO-1 expression. HUVEC were pre-incubated for 2 hous with dopamine (25 µM) or were untreated. Hereafter the cells were stored for 24 hours at 4°C. Proteins were isolated directly before and after cold storage, or 2 hours after rewarming. HO-1 was detected by Western blot analysis. For equal loading control, the membranes were stripped and re-probed with an anti-GAPDH antibody.*

B



***B**: HO-1 expression in HUVECs transfected with HO-1 siRNA. The expression of HO-1 was assayed 72 hours (3 days) or 120 hours (5 days) after transfection. Dopamine was either or not added at day 4 after transfection. Expression of HO-1 and GAPDH was assessed as described in A.*

C



***C**: Effect of dopamine on cold preservation injury in siRNA-transfected HUVEC. The cells were treated with HO-1 (+) or control (-) siRNA. Three days after transfection the cells were pre-incubated for 2 hrs with 25 µM of dopamine (hatched bars) or left untreated (open bars). LDH release was assessed after 24 hrs of cold storage. The results of a representative experiment (n=4) are depicted and expressed as mean LDH release ± SD.*

Discussion

Elucidation of structural requirements for substances to achieve protective activity against cold preservation damage of endothelial cells may both promote our understanding of the mechanisms involved in preservation injury of organ allografts and might eventually lead to the use of more effective and specific compounds for organ preservation. In this study, we analysed the protective potency of a series of substances that represent structural variations of the dopamine / dobutamine scaffold, compounds that have been shown earlier to protect against cold preservation injury both *in vitro* and *in vivo* (4-7, 14).

The variety of structures that are protective strongly disfavors the involvement of a receptor mediated process that would respond much more selective to the significant molecular modifications that were made. Moreover, the *in vivo* experiments where we could show that n-acetylation of dopamine completely abrogates its hemodynamic action are not in favor of receptor involvement. The only common feature that all protective compounds tested possess is the requirement for the presence of two hydroxyl groups at the benzene nucleus, located either in ortho or in para position. These isomers are known to be strong reducing agents due to the ease of quinone formation, which can not occur for the meta isomers. An additional requirement for protection is sufficient lipophilicity. As expected, the acylated dopamine derivatives enter cells more efficiently than the parent compound, but also seem to accumulate to a greater extent in mitochondria. Damage of mitochondria in endothelial cells is prevented by catecholamines as shown earlier (8). Therefore mitochondria might be considered as relevant targets for protection by catecholamines, although the functional relevance of an increased mitochondrial catecholamine uptake needs to be further defined. The increase in lipophilicity is evident upon acylation, because a nonpolar acyl chain is added and catecholamines lose their ability to form a cation. We are aware that the calculated logP value may differ from experimentally determined value, however, the calculated values for the catecholamines show significant agreement with published experimental data.

As logP refers to the uncharged compounds, the assumption that the importance of logP is embedded in a better cellular uptake may not be entirely true. Dihydroxybenzoic acids are present in their ionized forms at physiological pH, e.g. in the culture medium, which also might influence their cellular uptake. Within the series of uncharged amide compounds, the assumption is expected to be valid. The aryl derivatives 9 and 10 exhibit a slightly lower efficacy as expected from their logP value. However, they do not possess an alkyl chain that would allow them to insert into lipid membranes more efficiently.

Based on the structural requirement we postulate that insertion of the compounds into lipid compartments, which is underlined by the need for lipophilicity, might prevent oxidative damage at or near the membrane. Regardless of the detailed mechanism, an efficient protective substance requires an ortho- or para-dihydroxybenzene partial structure, is preferably uncharged with an alkyl chain and exhibits an overall lipophilicity exceeding a logP value of 3.

During hypothermic preservation an increase in the chelatable transition iron pool has been described to occur in a variety of cells (15-17). Catechols are able to form complexes with iron, which could further explain their protective effect. However, on the basis of our findings, this mechanism does not significantly contribute to protection. In most studies on mixed ligand complexes of iron, catechol yields the most stable complexes. Although meta- and para-compounds, i.e. resorcinol and hydroquinone (or their functional derivatives) usually result in approximately equally stable iron complexes, they yield much less stable iron complexes than the catechol compounds. In our study, the ortho- and para-compounds were both equally protective against cold perservation damage, while the meta-derivatives were not protective. Thus, the protective behavior does not match with the expected behaviour for formation of iron complexes.

Also the expression of HO-1, which has been shown to be beneficial with respect to cold preservation (13), seems not to be involved in the protective properties of lipophilic catechol containing compounds. In earlier studies, dopamine has been shown to induce HO-1 expression (18), but these concentrations were approximately 10 times higher compared to the concentrations required for protection against cold preservation injury. Nevertheless, lipophilic catechol containing compounds do preserve HO-1 expression during cold storage. This is an interesting aspect in the context of organ transplantation since HO-1 inhibits ischemia reperfusion injury (19, 20) and mediates anti-inflammatory effects (21, 22).

In conclusion, we have demonstrated that the protective effect of catecholamines on cold preservation injury is mediated exclusively by two structural entities, i.e. a strong reducing capacity and a minimal lipophilicity. These requirements can also be fulfilled in other dihydroxyphenolic compounds that lack hemodynamic action. Since acylated dopamine derivatives (23) and alkyl dihydroxybenzamides (24) have been described as anti-inflammatory agents, clinical use of such compounds in organ transplantaion seems to be a promising strategy not only to limit pre-transplantation injury in donor organs but also to limit the inflammatory response after transplantation.

References

1. Southard, J. H. & Belzer, F. O. (1995) Annu Rev Med 46, 235-47.
2. Shoskes, D. A. & Halloran, P. F. (1996) J Urol 155, 1831-40.
3. Haddad, P., Cabrillac, J. C., Piche, D., Musallam, L. & Huet, P. M. (1999) Cryobiology 39, 69-79.
4. Brinkkoetter, P. T., Beck, G. C., Gottmann, U., Loesel, R., Schnetzke, U., Rudic, B., Hanusch, C., Rafat, N., Liu, Z., Weiss, C., Leuvinik, H. G., Ploeg, R., Braun, C., Schnuelle, P., van der Woude, F. J. & Yard, B. A. (2006) Transplantation 82, 534-42.
5. Lindquist, J. M. & Rehnmark, S. (1998) J Biol Chem 273, 30147-56.
6. Schnuelle, P., Lorenz, D., Mueller, A., Trede, M. & Van Der Woude, F. J. (1999) Kidney Int 56, 738-46.
7. Schnuelle, P., Berger, S., de Boer, J., Persijn, G. & van der Woude, F. J. (2001) Transplantation 72, 455-63.
8. Yard, B., Beck, G., Schnuelle, P., Braun, C., Schaub, M., Bechtler, M., Gottmann, U., Xiao, Y., Breedijk, A., Wandschneider, S., Lösel, R., Sponer, G., Wehling, M. & van der Woude, F. J. (2004) Am J Transplant 4, 22-30.
9. Ahlenstiel, T., Burkhardt, G., Kohler, H. & Kuhlmann, M. K. (2003) Kidney Int 63, 554-63.
10. Krishna, C. M., Liebmann, J. E., Kaufman, D., DeGraff, W., Hahn, S. M., McMurry, T., Mitchell, J. B. & Russo, A. (1992) Arch Biochem Biophys 294, 98-106.
11. Freestone, P. P., Williams, P. H., Haigh, R. D., Maggs, A. F., Neal, C. P. & Lyte, M. (2002) Shock 18, 465-70.
12. Menasche, P., Grousset, C., Mouas, C. & Piwnica, A. (1990) J Thorac Cardiovasc Surg 100, 13-21.
13. Salahudeen, A. A., Jenkins, J. K., Huang, H., Ndebele, K. & Salahudeen, A. K. (2001) Transplantation 72, 1498-504.
14. Gottmann, U., Brinkkoetter, P. T., Bechtler, M., Hoeger, S., Karle, C., Schaub, M., Schnuelle, P., Yard, B., van der Woude, F. J. & Braun, C. (2006) Kidney Int 70, 321-8.
15. Rauen, U., Kerkweg, U., Wusteman, M. C. & de Groot, H. (2006) Cornea 25, 68-77.
16. Salahudeen, A. K. (2004) Am J Physiol Renal Physiol 287, F181-7.
17. Wyllie, S., Seu, P., Gao, F. Q. & Goss, J. A. (2003) Liver Transpl 9, 401-10.
18. Berger, S. P., Hunger, M., Yard, B. A., Schnuelle, P. & Van Der Woude, F. J. (2000) Kidney Int 58, 2314-9.
19. Liu, X., Pachori, A. S., Ward, C. A., Davis, J. P., Gnecchi, M., Kong, D., Zhang, L., Murduck, J., Yet, S. F., Perrella, M. A., Pratt, R. E., Dzau, V. J. & Melo, L. G. (2006) Faseb J 20, 207-16.
20. Wagner, M., Cadetg, P., Ruf, R., Mazzucchelli, L., Ferrari, P. & Redaelli, C. A. (2003) Kidney Int 63, 1564-73.
21. Freitas, A., Alves-Filho, J. C., Secco, D. D., Neto, A. F., Ferreira, S. H., Barja-Fidalgo, C. & Cunha, F. Q. (2006) Br J Pharmacol 149, 345-54.
22. Ferran, C. (2006) Transplantation 82, S36-40.
23. Loomans, M. E., Janusz, J. M. & Buckwalter, B. (1986) GB 2168976 .
24. Takita, H., Noda, S., Mukaida, Y., Inada, K., Toji, M., Kimura, F., Nitta, T., Watanabe, K., Umekawa, K. & Kobayashi, H. (1985) EP 0142241 .
25. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) J Clin Invest 52, 2745-56.
26. Fierz-David, H. E. & Kuster, W. (1939) Helv. Chim. Acta 22, 82-112.
27. Schröter, C. J., Braun, M., Englert, J., Beck, H., Schmid, H. & Kalbacher, H. (1999) J Immunol Methods 227, 161-8.

Chapter 5

Hypothermic preservation upregulates calpain expression and increases ubiquitination in vascular endothelial cells: influence of dopamine pre-treatment

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*: contributed equally to this work

Manuscript submitted

Abstract

Prolonged hypothermia, as occurs during solid organ transplantation, negatively influences transplantation outcome. Proteolysis is one of the deleterious events implicated in preservation injury of organ allografts. This strongly affects graft quality and hence immediate organ function. Since donor catecholamine treatment improves transplantation outcome after renal transplantation, the present study was conducted to examine the influence of dopamine (DA) pre-treatment on hypothermia induced proteolysis in endothelial cells subjected to prolonged cold storage.

HUVECs were highly susceptible to cold storage, which was reflected by morphological changes, loss of viability and by significant changes in cellular proteome. DA pre-treatment prevented cell death during cold storage. Western blot analysis demonstrated a time dependent up-regulation of calpain 1 and 2 during cold storage, which could be prevented by addition of EDTA. DA pre-treatment abolished auto-proteolysis of calpain 1. Analysis of ubiquitination revealed a significant increase in ubiquitinated conjugates after cold storage. This was not prevented by DA pre-treatment. Neither proteasome nor calpain inhibitors prevented cell death during cold storage.

In endothelial cells subjected to cold preservation, activation of the calpain pathway and the ubiquitin proteasome system occurs. Although DA pre-treatment inhibits the former, calpain inhibition did not protect endothelial cells during cold storage. DA pre-treatment might influence proteolysis, but proteolysis is not the major cause of endothelial cell death.

Introduction

Static cold storage is commonly used for organ preservation of allografts to bridge the time between organ explantation and implantation. Extended preservation times have allowed overcoming important logistical hurdles such as HLA-based organ sharing across geographical boundaries (1, 2). Being inexpensive, easy to apply and independent of technical devices, cold static storage is still the most practical way of organ preservation amongst the different preservation methods. However, prolonged cold ischemia time is associated with an increased rate of organ failure. Organ failure might occur as a consequence of endothelial barrier dysfunction (3-5), leading to parenchymal oedema, infiltration by polymorphonuclear leukocytes (PMNs) and ultimately to delayed graft function (6).

The use of organs from so called "marginal" donors has increased dramatically to meet the growing demand of organ allografts. Yet, organs obtained from these donors might be more susceptible to cold preservation injury, resulting in a higher rate of delayed graft function (7, 8) and possibly in a decreased allograft survival (9). Understanding the mechanisms that lead to tissue injury during hypothermic preservation is therefore of utmost importance to prevent organ damage and hence to improve graft quality.

Prolonged hypothermia is associated with cell and tissue damage as a consequence of necrosis or apoptosis (10). Apoptosis involves a complex network of enzymes that is tightly regulated by pro- and anti-apoptotic proteins, in which execution of cell death is mostly performed by caspases, calpains and cathepsins (11, 12). Although it is still a matter of debate whether apoptosis or necrosis are the major process leading to deterioration of graft quality during organ preservation, the use of anti-apoptotic drugs has shown encouraging results in pre-clinical and experimental studies (13).

Proteolyses is generally mediated by activation of a variety of enzymes. This might occur as a consequence of ATP depletion and an increase in cytosolic calcium. In particular, activation of the Ca^{2+}-dependent proteases, i.e. calpains, seems to be corollary to the imbalance in intracellular calcium homeostasis during cold storage (14, 15). Apart from elevated intracellular calcium concentrations, other mechanisms could also contribute to increase calpain activity, such as cleavage of calpastatin by caspases (16) or increased phospholipase activity (17).

The ubiquitin proteasome system (UPS) might be equally involved in proteolysis during cold preservation (18). It has been generally accepted that the most prominent function of the UPS

is to label cytosolic and nuclear proteins for proteosomal degradation. In addition, the UPS is involved in cell-cycle regulation, signal transduction and antigen processing (19-21).
Since we have previously reported that dopamine (DA) treatment of endothelial cells result in protection against cold preservation injury, in the present study we assessed to what extent hypothermia changes the cellular proteome and if DA treatment affects the major proteolytic pathways.

Material and methods

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as previously described (22). The cells were cultured in endothelial cell growth medium (EGM) (Promocell, Heidelberg, Germany) in T25 flasks (Greiner, Frickenhausen, Germany) coated with gelatin (1%). Confluent monolayers were passaged by Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO). Characterization of endothelial cells was performed on the basis of a positive uptake of acetylated LDL, a positive expression of Factor VIII related antigen and PECAM (CD31) (Dako, Glostrup, Denmark), and a negative staining for alpha smooth muscle actin. All experiments were performed with cell lines at passage three to seven.

Two-dimensional electrophoresis

Iso-electric focusing (IEF) was performed on precast 18 cm IPG strips with linear pH ranges using an IPGphor unit (Amersham Pharmacia, Frieburg, Germany). The strips were placed in ceramic strip holders and rehydrated in 350 µl rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3-10) containing 50 µg of protein extracts and protease inhibitors and a few grains of bromophenol blue. The strips were covered with 2 ml dry strip cover fluid (Amersham Pharmacia, Frieburg, Germany) and rehydrated over night by applying 30 V by constant temperature (20˚C) on the IPGphor unit. The following IEF protocol was used: 30 min gradient up to 500 V, 30 min 500 V, 30 min gradient up to 2000 V, 30 min 2000 V, 1 hr gradient up to 8000 V, 4 hrs 8000 V. After focusing the strips were washed with water and immediately used for the second dimension. Before loading on SDS-polyacrylamide gels (PAGE), the strips were equilibrated for 20 min in equilibration buffer containing iodoacetamide. Hereafter the strips were carefully washed with water and transferred to the SDS-PAGE. The gels were sealed on top with agarose sealing solution (0.5% low melting agarose in 25 mM Tris, 192 mM glycine and 0.1% SDS). Electrophoresis was carried out over

night in 25 mM Tris, 192 mM glycine and 0.1% SDS at 15˚C, applying a constant voltage of 100 V.

Tryptic digestion

Protein spots were excised from the gel and washed twice with deionized water, acetonytril (ACN)/water (1:1, v/v) and ACN. Proteins were digested overnight at 37˚C with 165 to 330 ng sequencing grade trypsin (Promega, Mannheim, Germany) in 40 mM ammonium bicarbonate. The reaction was stopped by freezing.

MALDI-TOF MS

MALDI-TOF mass spectra were recorded in the positive ion reflector mode with delayed extraction on a Reflex II TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a SCOUT 26-inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 26.5 kV, the reflector voltage was set to 30 kV and the first extraction plate was set to 20.6 kV. Mass spectra were obtained by averaging 200 to 300 individual laser shots. Calibration of the spectra was performed internally by two-point linear fit using autolysis products of trypsin. Sample preparation for MALDI mass fingerprints was achieved by co-crystallization of the matrix with ZipTip (Millipore, Schwalbach/Ts, Germany) concentrated samples. Database search was done against the NCBInr database using the MASCOT algorithm (www.matrixscience.com).

Measurement of ubiquitin-conjugates and free ubiquitin

HUVEC were incubated for 2hrs with 25µM of dopamine (DA, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). The culture flasks were washed 3 times with PBS (Invitrogen, Karlsruhe, Germany) and stored for different time period at 4°C in UW-solution. Hereafter the cells were collected using Trypsin/EDTA and centrifugation. The pellet was resuspended in 50 µl of distilled water to which a proteinase inhibitor cocktail (Roche, Mannheim, Germany) was added and immediately shock frozen in liquid nitrogen. The cells were subsequently lysed by 5 cycles of freezing in liquid nitrogen and quickly rewarming at 37˚C. After the final rewarming, the samples were centrifuged and the supernatants were frozen at -20˚C until use. Ubiquitin-conjugates and free ubiquitin were assessed by immunoblotting using rabit anti-ubiquitin antibody (Sigma, St. Louis, MO) and densitometric quantification of the chemo luminescence signal as described previously (23).

Western Blotting

HUVEC were harvested with trypsin/EDTA (Sigma-Aldrich, St. Louis, MO) and subsequently washed twice with cold PBS. The cells were lysed in lysis buffer (10 mM Tris, 2% SDS, 0.5% beta-mercaptoethanol) (all from Sigma-Aldrich, St. Louis, MO). Protein concentrations were measured using Coomassie-Reagent (Pierce, Rockford, USA). Samples (20 µg protein extract) were heated to 95°C for 5 min, loaded and separated on 10-20% SDS-polyacryamide gels followed by semi-dry blotted onto PVDF membranes (Roche, Mannheim, Germany). The membrane was blocked with blocking buffer (5% w/v nonfat dry milk in PBS-T) for 1h at room temperature. Thereafter, the blot was incubated with a polyclonal anti-calpain 1 or anti-alpain 2 (Cell Signaling Technology, Danvers, MA) or anti-ubiquitin antibody (Sigma, St. Louis, MO) overnight at 4°C. Subsequently the membrane was thoroughly washed with TBS-T and incubated with the appropriate horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch, Baltimore, MD) for 1 h at room temperature. Proteins were visualized using enhanced chemo luminescence technology according to the manufacturer's instructions (Pierce, Rockford, IL). To confirm equal protein loading, membranes were re-probed with a polyclonal anti-p42/p44 antibody (Cell Signaling Technology, INC., Rockland, Beverly, MA) or monoclonal anti-GAPDH antibody (Abcam, Cambridge, UK).

Measurements of intracellular Ca^{2+} concentrations

Measurements of intracellular Ca^{2+} concentrations were performed according to Koppel et al (24). HUVEC were incubated with phenol-red free medium (PRF) containing 4µm Fura-2 AM (Invitrogen, Karlsruhe, Germany) at 37°C for 1hr. The cells were washed with PBS twice and treated with dopamine for 2hrs. After washing the cells with PBS twice, PRF medium was added and the cells were subjected to 4°C for 3hrs. Coverslips were then mounted into a thermostatically regulated microscope chamber. A Zeiss Axiovert 35 (Zeiss, Hanau, Germany) inverted fluorescence microscope, equipped with a fluor 40/1.30 oil immersion objective and a charge-coupled device imaging camera (General Scanning, Planegg, Germany), was employed to detect fluorescence changes. Dual wavelength excitation at 340 and 380 nm was performed by an imaging system (Till Photonics, Planegg, Germany). After calibration, the following equation was used to relate the intensity ratios to cytosolic calcium levels: cytosolic calcium=Kd Q(R-Rmin)/(Rmax-R).

LDH assay

Lactate dehydrogenase assays were performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, HUVEC were plated in 24-well plates, grown until confluence and stimulated with 25µM dopamine (DA) for 2hrs. The plates were washed 3 times with 1ml of PBS and stored for 24hrs at 4°C in PRF medium. A 100µl aliquot of each supernatant was used to determine LDH release. In each experiment 100µl of PRF medium was used as blank. The results are expressed as OD490nm, corrected for the blank value.

Statistical analysis

Data are presented as mean ± SD for the indicated number of separate experiments. All analyses were based on more than three separate experiments. Differences between groups were determined by Student's *t* test. A *p*-value of less than 0.05 was considered statistically significant.

Results

We previously have demonstrated that hypothermic storage of endothelial cells severely affects cell viability, accompanied by profound changes in the expression of cytoskeleton proteins (5). To investigate to what extent hypothermia changes the cellular proteome, 2D-gel electrophoresis was performed (Fig 1). In general, we observed that there was a quantitative difference in the expression of a number of proteins when the cells were subjected to hypothermia for 24 hrs. A total of 50 spots were selected for further analysis. We were able to get protein sequence information in 47 of these spots. Quantitative analysis revealed that 16 different proteins were reduced in their expression (table1).

Because the reduction in protein expression during hypothermia might have been caused by proteolysis, we next investigated if the major proteolytic pathways, i.e. the caspase, calpain and proteosomal pathway, were activated during hypothermia. We did not observe activation of the caspase pathway as neither cleavage of the procaspases 8 and 9 nor that of caspase 3 occurred during hypothermia (data not shown). In contrast, activation of the calpain pathway was evident. This was reflected by an increase in the auto-proteolytic cleavage of calpain 1 (µ-calpain) during cold storage.

Figure 1A

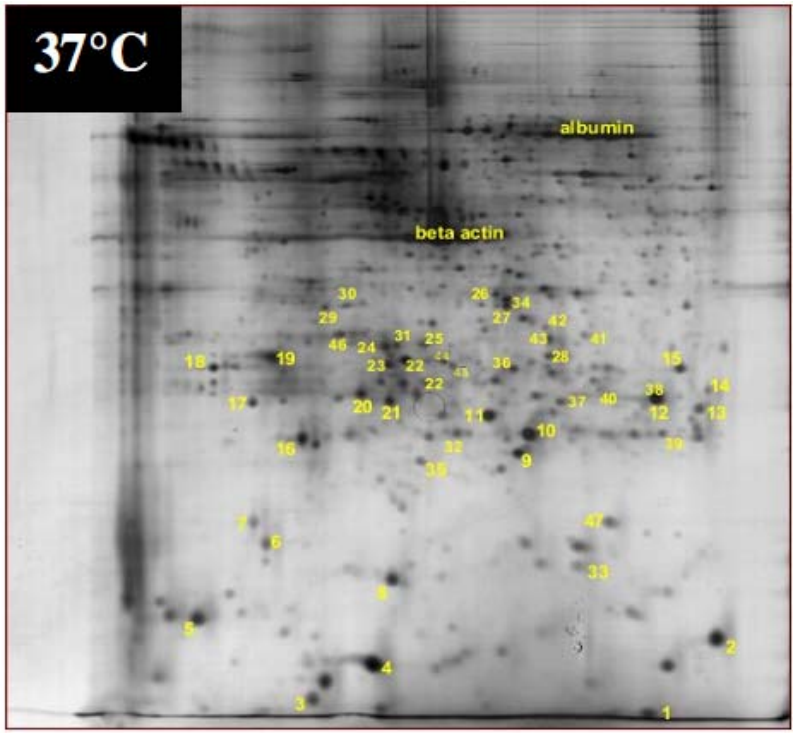

Figure 1B

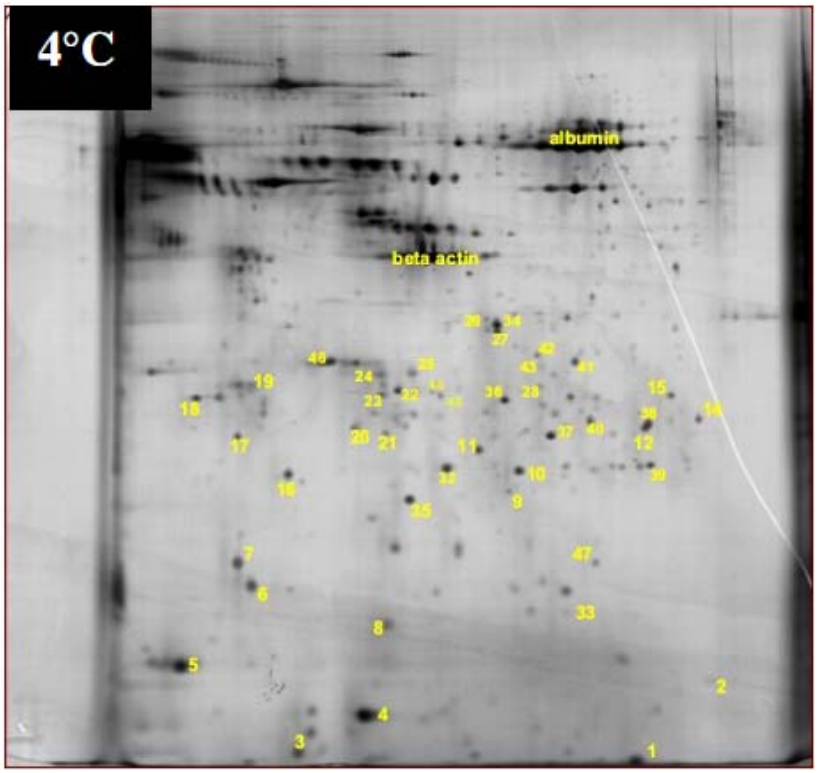

Figure 1 *Proteome analysis of HUVEC. Endothelial cells were either kept at 37˚C* ***(A)*** *or stored for 24 hrs at 4˚C in UW solution* ***(B).*** *Proteins were isolated and processed as described in materials and methods. Proteome analysis was performed for both conditions with three different HUVEC cultures. 2-D gel electrophoresis was performed for each*

individual sample. The result of a representative gels is shown. Numbers indicate the spots that were used for protein identification.

Dopamine pre-treatment completely prevented the cleavage of calpain 1 (Fig 2A). Calpain 2 (m-calpain) was upregulated already 6 hrs after the onset of cold storage. In endothelial cells that were treated with dopamine prior to hypothermia this was not observed (Fig 2B). Similarly, addition of EDTA prevented upregulation of m-calpain. Since the latter findings suggest that during cold storage an influx of extracellular calcium might occur which subsequently activates the calpain pathway, we assessed intracellular calcium concentration three hrs after the onset of cold storage in untreated and DA treated endothelial cells. Indeed, intracellular calcium concentrations were significantly increased after cold storage. This did not occur when the cells were pre-treated with DA or when EDTA was added to the preservation solution during cold storage (table 2).

Table 1: identification of proteins that were decreased in expression during cold storage

Spot number	protein
2	Fatty acid binding protein 5
8	Eukaryotic translation initiation factor 5
9	Thioredoxin B, Chain A
10	GST, Chain A
11	Ubiquitin carboxy-terminal hydrolase L1
12	Hsp27
13	Triosephosposphate isomerase
19	Cytoskeleton tropomyosin
26	F-actin capping protein alpha-1 subunit
29	Elongation factor 1 delta
30	Elongation factor 1 delta
31	Cathepsin D, Chain B
33	Oncoprotein 18
35	ATP synthase
37	Thioredoxin peroxidase
41	Protein pp44
44	Cathepsin D, preprotein
45	Cathepsin D, preprotein

In addition, to activation of the calpain pathway, protein degradation could occur via the UPS. Since protein ubiquitination is required for proteosomal degradation we determined if

hypothermia increased the amount of ubiquitin conjugates and free ubiquitin. The amount of free ubiquitin did not significantly change during hypothermia. However, the amount of ubiquitin conjugates was significantly increased. This was not influenced by dopamine pre-treatment (Fig 3).

Figure 2

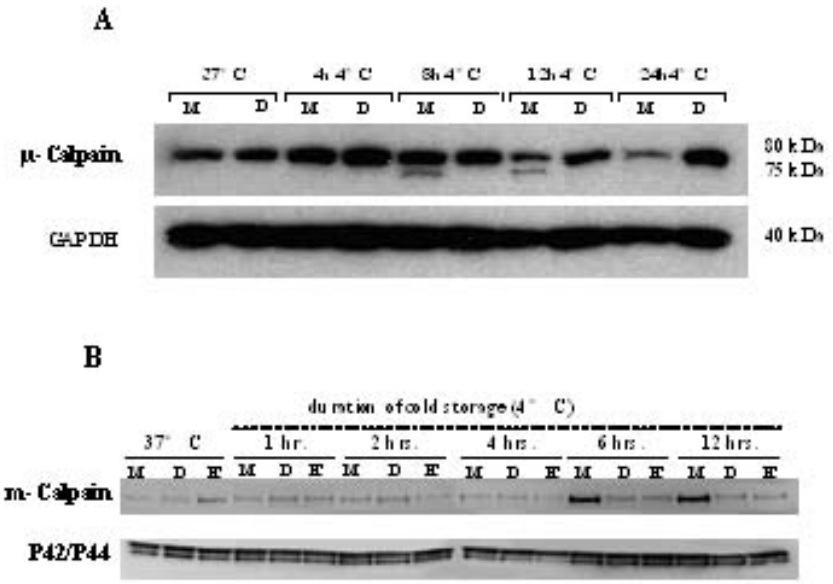

Figure 2 *Influence of hypothermia on calpain expression.* ***(A)*** *HUVEC were either treated for two hrs with dopamine (D, 25 μM) or left untreated (M). Hereafter the cells were stored for different time-period at 4˚C in University Wisconsin (UW) solution, or kept at 37˚C. Protein extracts were assessed for μ-calpain by means of Western blotting. P42/44 was used to demonstrate equal loading. Note that the 75 kDa auto-proteolytic fragment only occurs in untreated cells (M) stored at 4˚C. In addition, the expression of μ-calpain reduces with increasing cold storage time in untreated cells.* ***(B)*** *HUVECs were treated as in A. Apart from untreated (M) and dopamine treated (D) cells, EDTA (E) was added to the preservation solution during cold storage. Protein extracts were assessed for m-calpain by means of Western blotting. P42/44 was used to demonstrate equal loading. Note that the expression of m-calpain is increased in untread HUVEC. The results of a representative experiment is shown, a total of 4 different experiments were performed and yielded similar results.*

To test if activation of the calpain pathway and UPS were required for cell death during cold storage, endothelial cells were pre-treated with calpain inhibitor I, or the proteasome inhibitor lactacystine and stored at 4˚ C. The inhibitors were also present during cold preservation. Cell death was not prevented by these inhibitors as assessed by LDH release. The caspase 3 inhibitor ZVAD also did not prevent cell death. In contrast, LDH release was significantly inhibited by DA pre-treatment of the endothelial cells (Fig 4).

Table 2

Table 2: Intracellular Ca^{2+} concentrations after 3 hrs of hypothermia

	- EDTA	+ EDTA
Not treated	120 ± 42*	40 ± 10
Dopamine treated	39 ± 15	38 ± 12

*: Results are expressed as mean intracellular Ca^{2+} concentration (nM), *P*<0.01 not treated vs. Dopamine treated. *P*<0.01 –EDTA vs. +EDTA

Figure 3

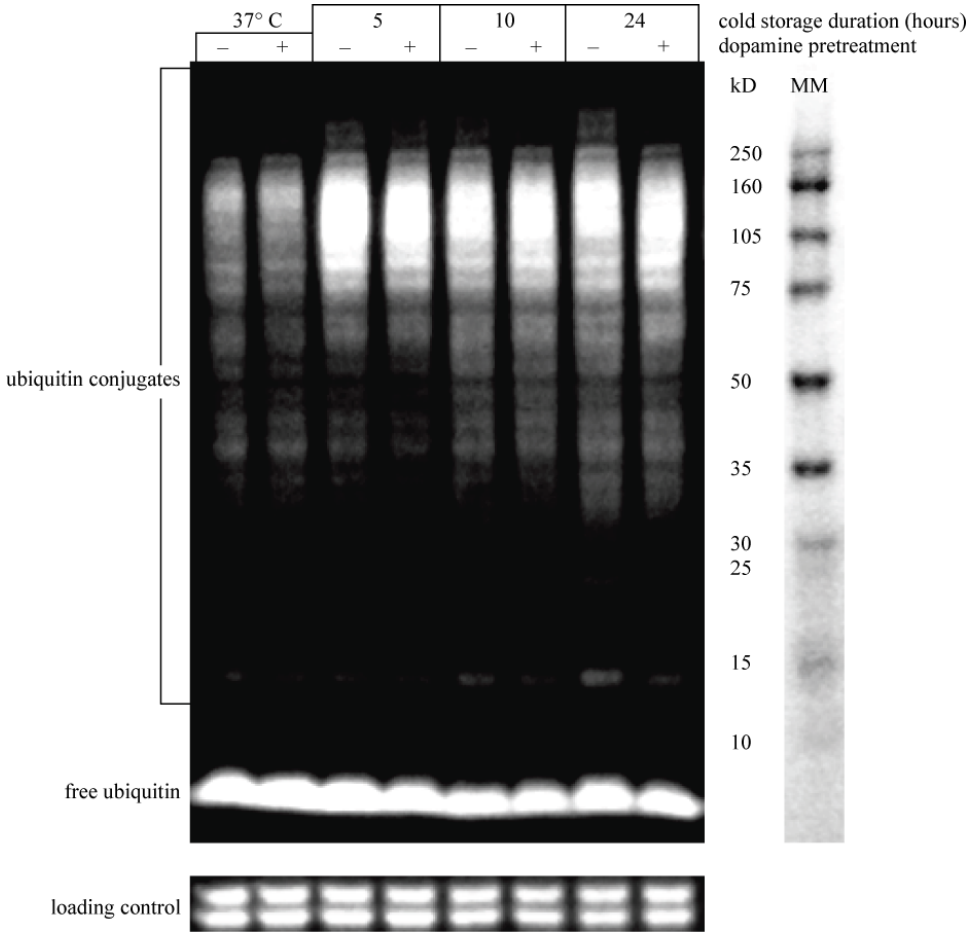

Figure 3 *Influence of hypothermia on free ubiquitin and ubiquitinated protein conjugates. HUVEC were treated for two hrs with 25 µM of dopamine (+) or left untreated (-). The cells were then stored at 4˚C in UW solution or kept at 37˚C in HUVEC medium. The result of a representative experiment is depicted (n=3).*

Figure 4

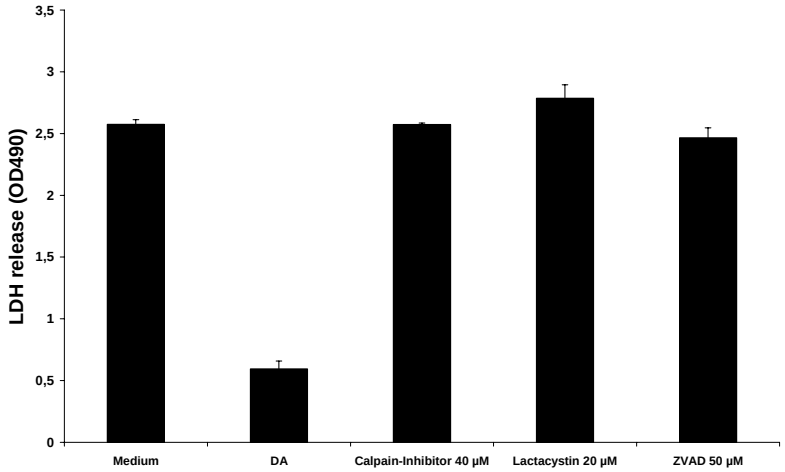

Figure 4 *Modulation of hypothermia mediated cell death. HUVEC were treated for 2 hrs with 25 µM of dopamine (DA), 40 µM of the calpain inhibitor I, 20 µM of the proteasome inhibitor lactacysteine or 50 µM of the caspase inhibitor ZVAD. Hereafter the cells were stored for 24 hrs at 4˚C in UW solution. With exception of dopamine, all inhibitors were present during cold storage. Untreated endothelial cells (Medium) were included in each experiment. A total of 5 different experiments were performed, the results are expressed as mean LDH release ± SD. (P<0.01 Medium vs. DA)*

Discussion

Although static cold storage is the most commonly used method for organ preservation, prolonged cold storage of allografts is associated with poor graft function (25, 26). Amongst the different mechanisms that might affect cell viability during cold storage, several studies have indicated that proteolysis might play an important role (5, 28). In previous *in vitro* and *in vivo* studies (25-27), we have shown that DA treatment significantly protects endothelial cells against hypothermia mediated cell damage. In the present study we assessed to what extent hypothermia influences the cellular proteome and if activation of proteolytic pathways during hypothermia was affected by DA treatment. The main findings of this study were as follows. Firstly, hypothermia leads to a reduction in the expression of a number of cellular proteins. Secondly, both the calpain and UPS pathway are activated during cold storage in HUVEC. No evidence for the activation of the caspase pathway was found. Thirdly, DA treatment affected activation of the calpain pathway, most likely by preventing influx of extracellular calcium. Increased ubiquitination was not prevented by DA treatment. Fourthly, inhibition of caspase-3, calpain or the proteasome did not prevent cell death during hypothermia.

Preservation injury is multifactorial and its mechanism is still incompletely defined. The complexity of preservation injury is further illustrated by the fact that different organs differ in tolerance against cold ischemia. Over the past decade however, a number of studies have shown that hypothermia-mediated cell damage can be overcome by appropriate treatment (29, 30). In particular the use of iron chelators or hydrophobic anti-oxidants has yielded encouraging results, showing their protective effect on different cell types (29-31). Yet, the mechanism by which cell damage occurs during cold storage might differ in detail between the cells that have been studied. Even when comparing the different studies using the same cell type, there are conflicting data in this regard. Although both Natori et al (32) and Sindram et al (33) have suggested that apoptosis is the major cause of sinusoidal endothelial cell death in liver allografts that are stored in UW solution, in the former study this was mediated by caspase activation while in the latter calpain activation was clearly involved. In our study using human umbilical vein endothelial cells we did not find activation of caspase during cold storage, in stead auto-proteolysis of μ-calpain and upregulation of m-calpain occurred. Activation of calpain might be a consequence of increased intracellular Ca^{2+} concentrations. During cold storage Ca^{2+} homeostasis is significantly affected because hypothermia most likely prolongs Ca^{2+} channel influx and alters ion exchangers that trigger enhanced sarcoplasmic reticulum (SR) Ca^{2+}-induced Ca^{2+} release. Depressed Ca^{2+}- and Na^{+}-pump activities may lead to Ca^{2+} and Na^{+} overload via Na^{+}-H^{+} exchange and reversed Na^{+}-Ca^{2+} exchange (34, 35).

The relevance of cellular Ca^{2+} influx for cell death during hypothermia has also been questioned. Recently Knoop et al (36) showed that cold storage of hepatocytes and endothelial cells in calcium-free media led to an increased cell injury whereas a physiological calcium concentration (2.5mM) was protective. These data do not corroborate with our own findings in that we clearly demonstrate that addition of EDTA not only prevented Ca^{2+} influx but also prevented cell death (our unpublished data). Similar findings were observed for DA treatment. It most be noted that both DA and EDTA have the propensity to chelate iron and thus we can not exclude that iron chelation is partly underlying this protection.

The UPS has also been implicated in cold preservation cell damage as has been reported by Majetschak M et al (18). In line with our data on endothelial cells, they found an increase in ubiquitin-protein conjugates in heart allografts subjected to cold storage. However, we could demonstrate that DA treatment did not prevent the increase in ubiquitin conjugates despite the fact that it protected endothelial cells during cold storage. It thus seems that proteosomal degradation of cellular proteins is not instrumental for cell death under these conditions. This

is further supported by the finding that lactacysteine could not prevent cell damage. It must also be stressed that cell death not necessarily depends on proteolysis, even as this might occur during prolonged hypothermia. As previously demonstrated, prolonged hypothermia results in severe structural changes (30) and functional deterioration of mitochondria (5), which will likely affects cell viability.

It remains to be assessed if differences in the cellular proteome that occur during cold storage are completely attributable to proteolysis. We have shown in previous studies that proteolysis at least occurs for some proteins, i.e. paxillin and vimentin (5), but loss in protein expression might equally be caused by cell death and subsequent release in the preservation solution.

In conclusion, our study demonstrates that hypothermia results in profound changes in the cellular proteome. Activation of the calpain pathway and UPS might occur during hypothermia, but inhibition of calpain or the proteosome could not prevent cell death. Although our data should not be generalized, as the mechanisms that lead to hypothermic cell death may differ for each cell type and model being studied, they clearly indicate that the protective effect of DA treatment is not related to the inhibition of major proteolytic pathways.

References

1. Southard JH, Belzer FO. Organ preservation. *Annu Rev Med* 1995; 46: 235.
2. McLaren AJ, Friend PJ. Trends in organ preservation. Transpl Int 2003; 16 (10): 701.
3. Lum H, Malik AB. Mechanisms of increased endothelial permeability. Can J Physiol Pharmacol 1995; 74: 787.
4. Carbognani P, Spaggiari L, Rusca M, et al. Pulmonary endothelial cell modifications after storage in solid-organ preservation solutions. J Int Med Res 1995; 23 (3): 200.
5. Brinkkoetter PT, Beck GC, Gottmann U et al. Hypothermia induced loss of endothelial barrier function is restored after dopamine pre-treatment: role of p42/p44 activation. Transplantation 2006; 82: 534-42
6. Morris PJ, Johnson RJ, Fuggle SV, Belger MA, Briggs JD. Analysis of factors that affect outcome of primary cadaveric renal transplantation in the UK. HLA Task Force of the Kidney Advisory Group of the United Kingdom Transplant Support Service Authority (UKTSSA). Lancet 1999; 354 (9185): 1147.
7. Nyhof E, Wiehl S, Steinhoff J, Krüger S, Mueller-Steinhardt M, Fricke L. Relationship between donor factors, immunogenic up-regulation, and outcome after kidney transplantation. Transplant Proc 2005; 37:1605-7
8. Parzanese I, Maccarone D, Caniglia L, Pisani F, Mazzotta C, Rizza V, Famulari A. Risk factors that can influence kidney transplant outcome. Transplant Proc 2006 38; 1022-3
9. Weimert NA, Alloway RR. Renal transplantation in high-risk patients. Drugs 2007; 67: 1603-27
10. Gujral JS, Bucci TJ, Farhood A and Jaeschke H, Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? Hepatology 2001; 33:397-405
11. Jani A, Ljubanovic D, Faubel S, Kim J, Mischak R, Edelstein CL. Caspase inhibition prevents the increase in caspase-3, -2, -8 and -9 activity and apoptosis in the cold ischemic mouse kidney. Am J Transplant 2004; 4: 1246-54
12. Sindram D, Kohli V, Madden JF, Clavien PA. Calpain inhibition prevents sinusoidal endothelial cell apoptosis in the cold ischemic rat liver. Transplantation 1999; 68 (1): 136.
13. Hoglen NC, Anselmo DM, Katori M et al. A caspase inhibitor, IDN-6556, ameliorates early hepatic injury in an ex vivo rat model of warm and cold ischemia. Liver Transpl. 2007 Mar;13(3):361-6
14. Burns AT, Davies DR, McLaren AJ, Cerundolo L, Morris PJ, Fuggle SV. Apoptosis in ischemia/reperfusion injury of human renal allografts. Transplantation 1998; 66 (7): 872.
15. Kohli V, Gao W, Camargo CA, Jr., Clavien PA. Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. Proc Natl Acad Sci U S A 1997; 94 (17): 9354.
16. Goll DE, Thompson VF, Li H, Wei W, Cong J. The Calpain System. Physiol Rev 2003; 83: 731.
17. Wang KK, Posmantur R, Nadimpalli R, et al. Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch Biochem Biophys 1998; 356 (2): 187.
18. Majetschak M, Patel MB, Sorell LT, Liotta C, Li S, Pham SM. Cardiac proteasome dysfunction during cold ischemic storage and reperfusion in a murine heart transplantation model. Biochem Biophys Res Commun 2008; 365(4):882-8.
19. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998; 67: 425.
20. Ciechanover A. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. 1998; 17 (24): 7151.
21. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82 (2): 373.
22. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52 (11): 2745.
23. Ponelies N, Hirsch T, Krehmeier U, Denz C, Patel MB, Majetschak M. Cytosolic ubiquitin and ubiquitylation rates in human peripheral blood mononuclear cells during sepsis. Shock 2005; 24: 20-5
24. Koppel H, Yard BA, Christ M, Wehling M, van der Woude FJ. Modulation of angiotensin II-mediated signalling by heparan sulphate glycosaminoglycans. Nephrol Dial Transplant 2003; 18: 2240-2247
25. Schnuelle P, Yard BA, Braun C, et al. Impact of donor dopamine on immediate graft function after kidney transplantation. Am J Transplant 2004; 4: 419.
26. Gottmann U, Notheisen A, Brinkkoetter PT, et al. Influence of donor pretreatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 2005; 79 (10): 1344.
27. van der Woude FJ, Schnuelle P, Yard BA. Preconditioning strategies to limit graft immunogenicity and cold ischemic organ injury. J Investig Med 2004; 52 (5): 323.
28. Charrueau C, Blonde-Cynober F, Coudray-lucas C et al. Prevention of proteolysis in cold-stored rat liver by addition of amino acids to the preservation solution. J Gastroenterol Hepatol 2000; 15: 1199-204.
29. Rauen U, Kerkweg U, Wusteman MC, de Groot H. Cold-induced injury to porcine corneal endothelial cells and its mediation by chelatable iron: implications for corneal preservation. Cornea 2006; 25: 68-77.
30. Yard B, Beck G, Schnuelle P et al. Prevention of Cold-Preservation Injury of Cultured Endothelial Cells by Catecholamines and Related Compounds. Am J Transplant 2004; 4: 22-30

31. Rauen U, Kerkweg U, de Groot H. Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions. Cryobiology 2007; 54: 77-86
32. Natori S, Higuchi H, Contreras P, Gores GJ. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. Liver Transpl 2003; 9(3):278-84
33. Sindram D, Kohli V, Madden JF, Clavien PA. Calpain inhibition prevents sinusoidal endothelial cell apoptosis in the cold ischemic rat liver. Transplantation 1999; 68(1):136-40
34. Puglisi JL, Bassani RA, Bassani JW, Amin JV, Bers DM. Temperature and relative contributions of Ca transport systems in cardiac myocyte relaxation. Am J Physiol 1996; 270:H1772–H1778
35. Sutko JL. The effect of temperature on Ca2+ uptake and Ca2+ activated ATP hydrolysis by sarcoplasmic reticulum. Experientia 1973; 29:396–398
36. Knoop S, de Groot H, Rauen U. Little evidence for a major role of Ca2+ in cold-induced injury of liver cells. Cryobiology 2008; 56(2):103-13

Chapter 6

HMGB1 and adenosine are both released by endothelial cells during hypothermic preservation

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Abstract

Hypothermic preservation of solid allografts causes profound damage of vascular endothelial cells. This in turn might activate innate immunity. In the present study we employed an *in vitro* model to study to what extent supernatants of damaged endothelial cells are able to activate innate immunity and to study the nature of these signals. The expression of HMGB1 and adhesion molecules on HUVEC was studied by immunofluorescence, FACS and Westernblotting. Cytokine production was performed by ELISA. HMGB1 expression was completely lost in endothelial cells after hypothermic preservation. This was associated with cell damage as it only occurred in untreated endothelial cell but not in cells rendered resistant to hypothermia-mediated damage, by dopamine treatment. Only supernatants from hypothermia susceptible cells up-regulated the expression of IL-8 and adhesion molecules in cultured endothelial cells in an HMGB1-dependent manner. In whole blood assays, both supernatants of hypothermia susceptible and resistant cells inhibited TNFα production, concomitantly with an increased IL-10 secretion. The activity of the supernatants was found already after 6 hrs of hypothermic preservation, and paralleled the decrease in intra-cellular ATP levels. Modulation of TNFα and IL-10 production by these supernatants was completely abrogated by prior treatment with adenosine deaminase and was similar to the response of an A2R agonist. Our study demonstrates that both HMGB1 and adenosine are released during hypothermic preservation. While release of HMGB1 is caused by cell damage, release of adenosine seems to be related to ATP hydrolysis, occurring in both susceptible and resistant cells.

Introduction

Allocation of organ allografts has become possible by adequate improvements in organ preservation. In transplantation of solid organs, static cold storage is the most widely used modality for organ preservation. Yet, acute shortage of donor organs and the inevitable pressure to use sub-optimal donors, has made this method questionable to prevent deterioration of organ quality (1, 2). Prolonged hypothermic preservation remains a significant cause of pre-tranplantation injury of allografts. Because pre-tranplantation injury significantly affects short and long term organ function (3-5), cold ischemia time is a critically important factor for transplantation outcome (3, 4). Tissue injury caused by hypothermia is largely mediated by oxidative stress (6-8). Injury to endothelial cells may lead to impaired control of vascular permeability (9), thrombosis (10) and inflammation (10, 11).

According to the danger hypothesis (12), tissue damage might evoke immune activation and therefore tissue damage seems to increase tissue immunogenicity of the allograft. Although this theory has now widely been accepted, the signals that initiate immune activation and modulate innate immunity are not clearly defined in organ transplantation.

High mobility group box chromosomal protein 1 (HMGB1) is a highly conserved chromatin binding protein which is abundantly expressed in mammalian tissues (13). In addition to its crucial roles in stabilizing nucleosome formation, facilitating gene transcription and modulating steroid hormone receptors, its role in innate immunity has been widely appreciated (13-15). HMGB1 can be passively released by necrotic cells (16) or actively secreted by monocytes, macrophages and dendritic cells via a non-classical vesicle-mediated secretory pathway (17-19). The presence of extracellular HMGB1 is not only a telltale that a cell or tissue has suffered from damage, but also provides “danger” signals to a variety of cells that constitute the innate immune system (14, 20, 21). Binding of HMGB1 to the receptor for advanced glycation end products (RAGE) on endothelial cells and monocytes increases the expression of adhesion molecules and stimulates the production of an array of pro-inflammatory cytokines (22, 23). The involvement of Toll like receptor (TLR) 2 and 4 in cell activation by HMGB1 has also been demonstrated (24).

We and others have previously shown that cold preservation of endothelial cells results in a rapid loss of endothelial barrier function (9) and induces necrosis of these cells (25-27). Therefore, cold preservation of allografts might be accompanied by the release of HMGB1 from endothelial cells and hence might contribute to activation of the innate immune system and subsequently to inflammation (13-15, 22, 23).

Apart from signals that activate innate immunity, other factors might be present that downregulate inflammation. In particular adenosine, a purine nucleoside that is released extracellularly, has a potent endogenous anti-inflammatory potential (28). Under circumstances, such as ischemia, a rapid and massive degradation of intracellular ATP eventually leads to accumulation of AMP, which in turn, is converted to adenosine (29, 30). Also during cold preservation of organ allografts, intracellular ATP is depleted within hours**,** an eligible condition for extracellular release of adenosine. It is therefore plausible that adenosine release during hypothermic preservation of allografts is another pivotal modulator of the immune response in the recipient.

In the present study, we used an *in vitro* model to investigate if hypothermic preservation of endothelial cells is associated with the release of HMGB1 and adenosine. In addition, the functional relevance of these factors, in terms of immune activation was studied.

Materials and methods

Reagents

Endothelial cell growth MedKIT, Phenol-red free medium (Promocell, Heidelberg, Germany), PBS, RPMI1640 (GIBCO, invitrogen, NY), Fetal bovine serum (FBS) Gold (PAA laboratories GmbH, Pasching, Austria), Tripsin/EDTA solution, LPS from E.coli, CGS 21680, DMSO, TritonX-100 (Sigma, St. Louis, MO), Bovine Serum Albumin (SERVA, Heidelberg, Germany), 37% formaldehyde (Mallinckrodt Baker, Deventer, Holland), Ficoll-paqueTM plus (Amersham Biosciences AB, Uppsala, Sweden), PGN-BS (B. subtilis), Pam3CSK4, Poly (I:C), Flagellin (B. subtilis), CL087, ssRNA40/LyoVec, ODN2006 (Invitrogen, San Diego, CA). ZM241385 (Tocris Cookson Inc., Ellisville, MO), HMG1 (Proteinone, Bethesda, MD).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords as described previously (26). The cells were grown in basal endothelial medium supplemented with 10% FBS and essential growth factors until they formed a confluent monolayer. Cold preservation was performed essentially the same as described (26). In brief, 2 hrs before cold preservation was started HUVECs were either treated with Dopamine (DA, 30 μM) or left untreated. The cells were stored at 4˚C in Phenol-red free (PRF) cell-culture medium or preservation solution (university of Wisconsin, UW) depending on the specific experiment.

Immunohistochemical staining

HMGB1 staining was performed on HUVECs and peripheral blood mononuclear cells (PBMC). HUVECs were cultured on glass coverslips until confluence. The cells were treated for 2 hrs with DA (30μM) or left untreated and stored for 24 hrs at 4˚C in UW solution. Cells that were cultured for the same period of time at 37˚C were included in each experiment. PBMCs were isolated by Ficoll-Paque plus density-gradient centrifugation and mounted on glass by cytospin centrifugation. All cells were fixed in 4% freshly-made formaldehyde for 1hr at room temperature followed by permeabilization with ice-cold 0.5% Triton X-100 for 5 min. The cells were then incubated for 30 min. at room temperature with PBS containing 2% BSA to block unspecific background staining. A polyclonal rabbit anti-HMGB1 antibody (Biomol., Lakeplacid, NY,) was added overnight to the samples at 4˚C. After extensively washing with PBS/BSA the samples were incubated for 1hr at room temperature with a Texas-red-conjugated goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) or FITC conjugated swine anti-rabbit IgG (Dako, Hamburg, Germany). Cells were incubated with DAPI or TOTO3 (Molecular Probes, Leiden, The Netherlands) for 15 min at room temperature to visualize nuclei. Fluorescence signals were analyzed by fluorescence and confocal microscopy.

Assessment of intracellular ATP

Confluent HUVEC monolayers were treated for 2hrs with 30 μM dopamine or left untreated. Subsequently the cells were washed and stored at 4°C. Intracellular ATP was extracted at serial time points and measured by luciferase driven bioluminescence using the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany).

Activation of endothelial cells by supernatants

To test if the supernatants of endothelial cells that were stored for 24 hrs at 4ºC contain biological activity, normal cultured HUVEC were incubated for 24 hrs at 37ºC with the supernatants in a 1:1 dilution. Hereafter IL-8 production was measured by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Beckman Coulter, Marseille Cedex, France). HMGB1 specificity in the supernatants was tested by immune-absorption. To this end, HMGB1 by adding a polyclonal rabbit anti-HMGB1 antibody (Biomol., Lakeplacid, NY) or normal rabbit IgG was added for 1 hr followed by the addition of protein A/G (1 hr) (Santa Cruz, Heidelberg, Germany). The supernatants were centrifuged and used for further experiments. Absorption of HMGB1 was demonstrated by Westernblot

(data not shown). Apart from IL-8 production activation of HUVEC by the supernatants was also tested by FACS analysis as described below.

FACS analysis

HUVECs were cultured in medium supplemented with supernatants (dilution 1:1) from endothelial cells that were subjected to cold preservation. FACS analysis was performed with 2×10^6 cells using the following FITC conjugated monoclonal antibodies; anti-human ICAM-1, anti-human VCAM-1 and anti-human E-selectin (all from Becton Dickinson, Heidelberg, Germany). Antibodies were added for 30 min at 4ºC followed by extensively washing with PBS. FACS analysis was performed on a FACScalibur equipped with the CELLQuest software (Becton Dickinson, Heidelberg, Germany). The data were analysed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

Whole blood assay

Blood was obtained from healthy adult donors with informed consent and diluted 1:1 with PRF medium or with supernatants obtained from HUVECs that were stored for 24 hrs at 4˚C in PRF medium. HUVECs were either left untreated or treated with DA (30 µM) 2 hours prior to cold storage as described above. The diluted blood was subsequently plated in 24 well plates containing either culture medium or one of the following stimuli: 20µg/ml PGN-BS (B. subtilis), 10µg/ml Pam3CSK4, 50µg/ml Poly (I:C), 0.5µg/ml LPS, 1µg/ml Flagellin (B. subtilis), 1µg/ml CL087, 1µg/ml ssRNA40/LyoVec, 1µM ODN2006 (Invivogen, San Diego, CA). In some experiments, recombinant HMGB1 (0.1µg/ml) (Proteinone, Bethesda, MD) or the adenosine receptor A2A agonist CGS 21680 (5µM) (Sigma, St. Louis, MO) was added. The concentrations of different TLR ligands were based on a previous publication (31) and product data sheet. The plates were incubated for 24hrs at 37˚C (5% CO_2/ 95% humidity) after which supernatants were harvested and assessed for TNFα and IL-10 production. TNFα and IL-10 concentrations the supernatant after whole blood stimulation were measured by ELISA (both from Beckman Coulter, Marseille Cedex, France) according to the manufacturer's instructions.

Westernblot analysis

Supernatants from HUVECs that were subjected to cold preservation for 24hrs were analysed for the presence HMGB1 by western blot. The supernatant were 50x concentrated by means of centricon columns (Millipore, Billerica, MA). The presence of HMGB1 in cell nuclei

isolated from the same HUVEC was also analysed. SDS-PAGE and Westernblotting was performed essentially similar as described (26). Briefly, samples were resolved on 10 % SDS-PAGE and electrotransferred onto PVDF filters. Hereafter the filters were incubated overnight with 5% non-fat dry milk powder in PBS to block unspecific background staining. A polyclonal rabbit anti-HMGB1 antibody (Biomol., Lakeplacid, NY) followed by a horseradish peroxidise-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany) (both dissolved in PBS/0.2 % Tween-20/5 % non-fat dry milk powder) were subsequently added for 60 minutes. Visualization of immunoreactive bands was performed by chemiluminescence reagent (PerkinElmer LAS Inc., Boston, MA) according to the manufacture's instructions.

Statistical analysis

Data were presented as mean ± SD for the indicated number of separate experiments. All analyses were based on more than three separate experiments. Differences between groups were determined by Student's T test. A *p*-value of less than 0.05 was considered statistically significant.

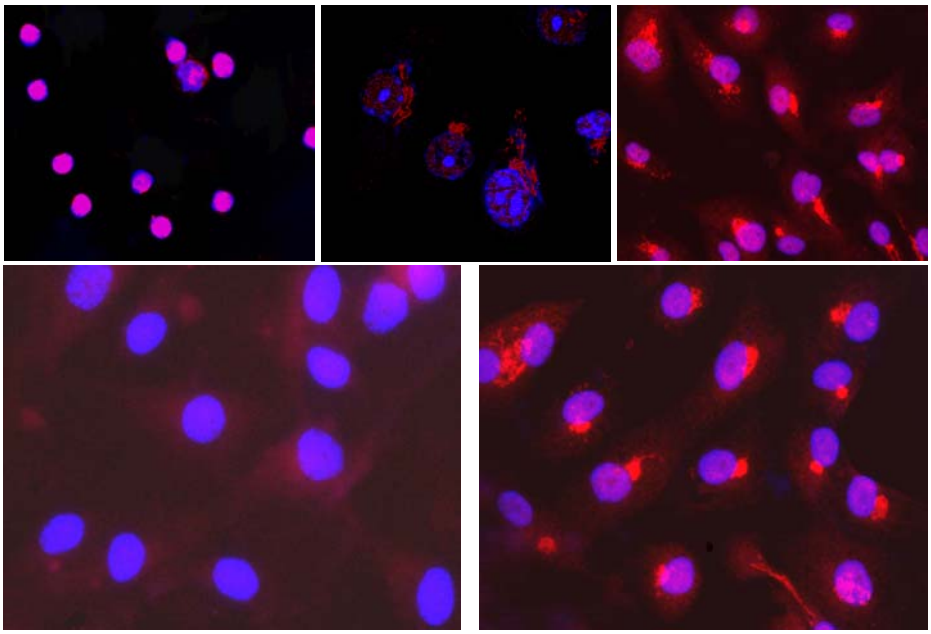
Results

Release of HMGB1 during cold preservation

The expression of HMGB1 was investigated by means of immunefluorescence. In cultured HUVEC, HMGB1 expression was found both in the nuclei and in an extra-nuclear compartment in close vicinity to the nucleus (Fig. 1, upper panel to the left). By using confocal immunefluorescence microscopy extra-nuclear HMGB1 expression could be confirmed (Fig. 1A, upper panel, in the middle). Extra-nuclear HMGB1 expression was only found in HUVEC and not in PBMC (Fig. 1A, upper panel to the right). In untreated HUVEC that were subjected to 24 hrs of cold preservation, the extra-nuclear compartment was completely lost, while nuclear staining of HMGB1 was strongly diminished (Fig. 1A, lower panel to the left). Cold-preservation of untreated HUVEC was characterized by release of LDH, which was completely abrogated by 2 hours of dopamine treatment prior to cold preservation (data not show). Under the latter condition, loss of HMGB1 expression was not observed (Fig. 1A, lower panel to the right). Westernblot analysis of isolated nuclei and supernatants confirmed the loss of HMGB1 in nuclei of untreated cells concomitantly with the appearance of the protein in the supernatant (Fig. 1B).

Figure 1

A.



B.

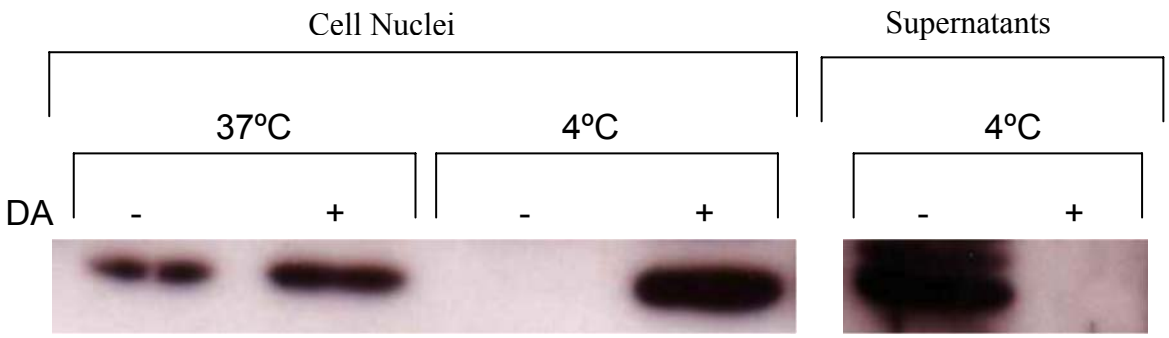
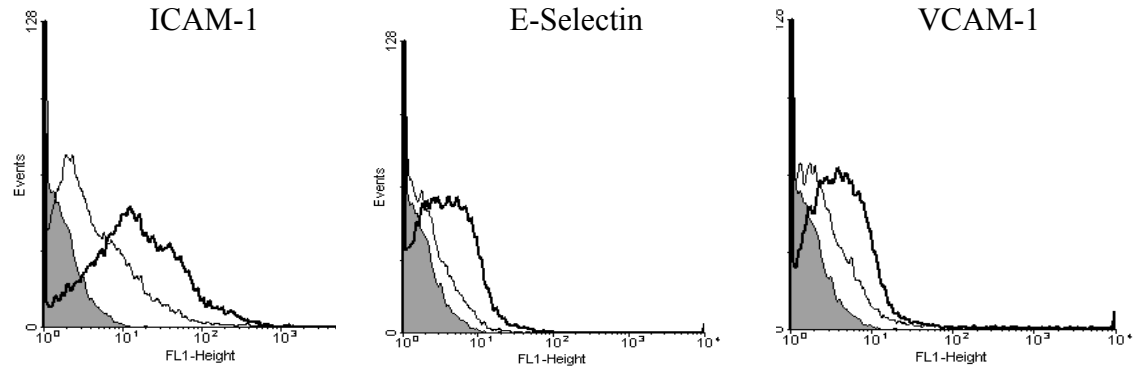


Figure 1 A: *HMGB1 expression in endothelial cells. Cultured HUVEC (upper two panels to the left) and freshly isolated PBMC (upper panel to the right) were stained for HMGB1 as described in the materials and methods section. Analysis of HMGB1 was performed by means of immune-fluorescence (upper panels to the left and to the right) and by confocal microscopy. Original magnification: 200x and 400x respectively. The lower panels show HMGB1 expression in untreated HUVEC (to the right) and dopamine treated HUVEC (to the left after 24 hrs of cold storage. Original magnification: 400x.* ***B:*** *Westernblot analysis of HMGB1 in cell nuclei from HUVEC before (37˚C) and after cold storage (4˚C). Note the disappearance of HMGB1 from the nuclei after cold storage of untreated HUVEC (-) and its appearance the supernatant of these cells. Da: dopamine 30 µM*

Figure 2

A.



B.

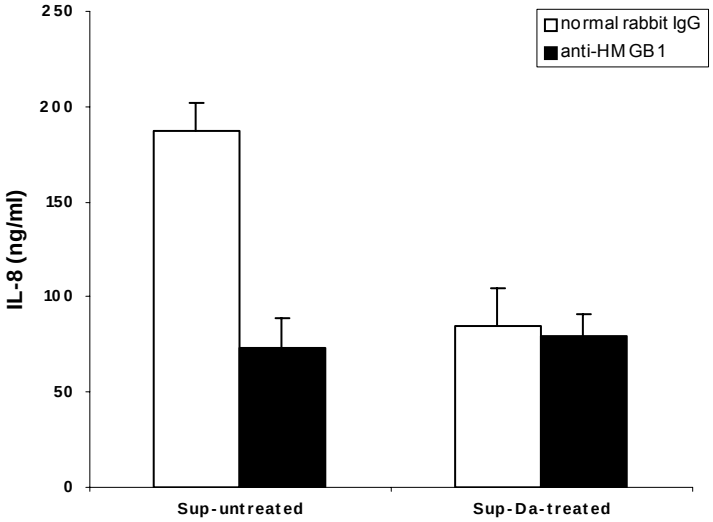


Figure 2 *Upregulation of adhesion molecules and IL-8 production by supernatants.* ***A:*** *Normal cultured endothelial cell were incubated for 24 hrs with supernatants obtained from untreated (bold line) and dopamine treated (thin line) HUVEC that were subjected to 24 hrs of cold storage. Note that supernatants from untreated HUVEC up-regulates the expression of ICAM-1, E-selectin and VCAM-1 compared to supernatants from dopamine treated HUVEC. Filled histogram represents the negative control. The results from a representative experiment (n=5) is depicted.* ***B:*** *Normal cultured endothelial cell were incubated for 24 hrs with supernatants obtained from untreated (Sup-untreated) and dopamine treated (Sup-Da-treated) HUVEC that were subjected to 24 hrs of cold storage. The supernatants were immune-absorbed with anti-HMGB1 (filled bars) or normal rabbit IgG as described in the materials and methods section. IL-8 production was measured by ELISA using triplicates for each condition. The results are expressed as mean IL-8 production (ng/ml) ± SD from 4 different experiments.*

Modulation of adhesion molecules and IL-8 expression in cultured endothelial cells

To test if the release of HMGB1 from unprotected HUVEC has functional significance, cultured endothelial cells were stimulated with supernatants of untreated or dopamine treated HUVEC for 24 hrs, after which the expression of ICAM-1, E-selectin and VCAM-1 was determined. The expression of all three adhesion molecules was upregulated by supernatants obtained from untreated HUVEC (Fig. 2A). Similarly, IL-8 production was two times higher in endothelial cell cultures stimulated with supernatants of untreated HUVEC. This was due to the release of HMGB1 since depletion of HMGB1 from these supernatants by means of immune-absorption completely normalized IL-8 production (Fig. 2B).

Modulation of TNFα and IL-10 production

We next investigated if supernatants obtained from HUVEC after cold storage are able to modulate LPS mediated TNFα and IL-10 production in whole blood assays. Both supernatants from untreated and dopamine treated HUVEC (n=6) consistently suppressed TNFα production, while the production of IL-10 was increased. This was unlikely mediated by HMGB1 since addition of recombinant HMGB1 augmented both TNFα and IL-10 production (Fig. 3).

Figure 3

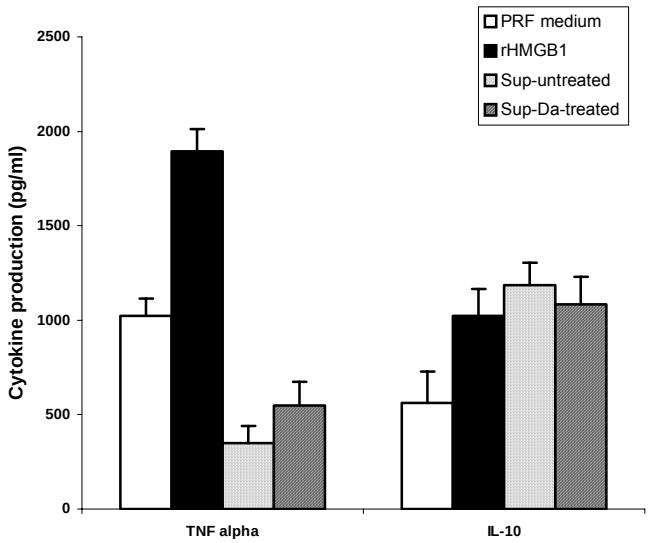


Figure 3 *LPS mediated TNFα and IL-10 production in whole blood assays. Whole blood was diluted 1:1 in normal PRF medium to which either nothing (open bars) or 0.1 µg/ml recombinant HMGB1 (filled bars) was added. In addition, whole blood was diluted 1:1 with supernatants obtained after cold storage of untreated HUVEC (Sup-untreated; dotted bars) or dopamine treated (Sup-Da-treated; hatched bars). To each condition 0.5 µg/ml of LPS was added. TNFα and IL-10 production was assessed after 24 hrs by ELISA, using triplicates for each condition. A total of 6 experiments were performed with different blood donors and different HUVEC supernatants. The result of a representative experiment is depicted.*

Although upregulation in TNFα and IL-10 production was also observed when other TLR ligands were added to the whole blood assay, inhibition of TNFα production by supernatants obtained from HUVEC after cold storage only occurred in a TLR4 dependent fashion (Fig. 4). This was also found for the upregulation in IL-10 productiom. Supernatants from dopamine treated and untreated HUVEC behaved similar in this regard (data not shown).

Figure 4

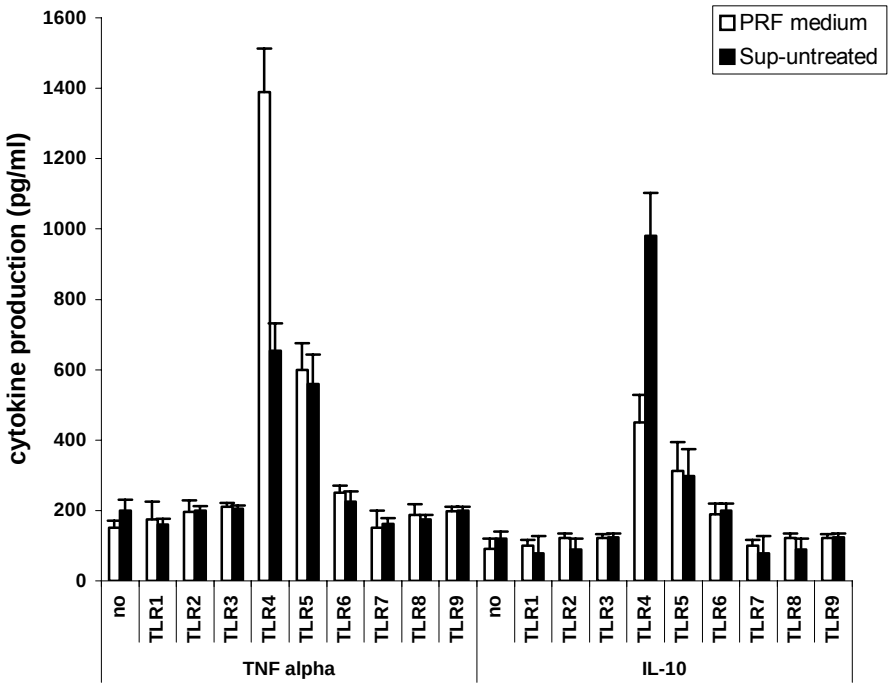


Figure 4 *Modulation of TLR-mediated TNFα and IL-10 production. Whole blood was diluted 1:1 in normal PRF medium (open bars) or in supernatants of untreated HUVEC (Sup-untreated), obtained directly after 24 hrs of cold storage. To the wells either nothing (no) or different TLR ligands (TLR 1-9) was added. The different ligands that were used were as follows: PGN-BS (20µg/ml) (TLR1), Pam3CSK4 (10µg/ml) (TLR2), Poly (I:C) (50µg/ml) (TLR3), LPS 500 ng/ml (TLR4), Flagellin (1µg/ml) (TLR5), CL087 (1µg/ml) (TLR6), ssRNA (1µg/ml) (TLR8) and ODN2006 (1µM) (TLR9). TNFα and IL-10 production was assessed as described in figure 3. A total of 4 different experiments were performed. The result of a representative experiment is expressed as mean cytokine production (pg/ml) ± SD.*

The modulatory activity found in the supernatants was present already 6 hrs after cold storage and coincided with loss of intra-cellular ATP levels (Fig. 5). Although intracellular ATP levels further decreased in time during cold preservation, modulation of TNFα and IL-10 production was maximal after 6 hrs. In subsequent experiments the involvement of adenosine, a product of ATP hydrolysis, in modulation of cytokine production was studied. When whole

blood assays were performed in normal phenol red free (PRF) medium, addition of the non-specific adenosine A2A and A2B receptor agonist NECA significantly inhibited TNFα production, while IL-10 production was significantly up-regulated to a similar extent as supernatants from HUVEC after cold storage (Fig. 6A). The specific adenosine A2A receptor agonist CGS 21680 did not differ from NECA in this regard (data not shown). Addition of NECA HUVEC supernatants did not influence TNFα or IL-10 production (Fig. 6A). In contrast, addition of adenosine deaminase (ADA) completely abrogated the modulatory effect of the supernatants, while addition of ADA to normal PRF medium did not influence the production of TNFα or IL-10 in whole blood assays (Fig. 6B).

Figure 5

A. **B.**

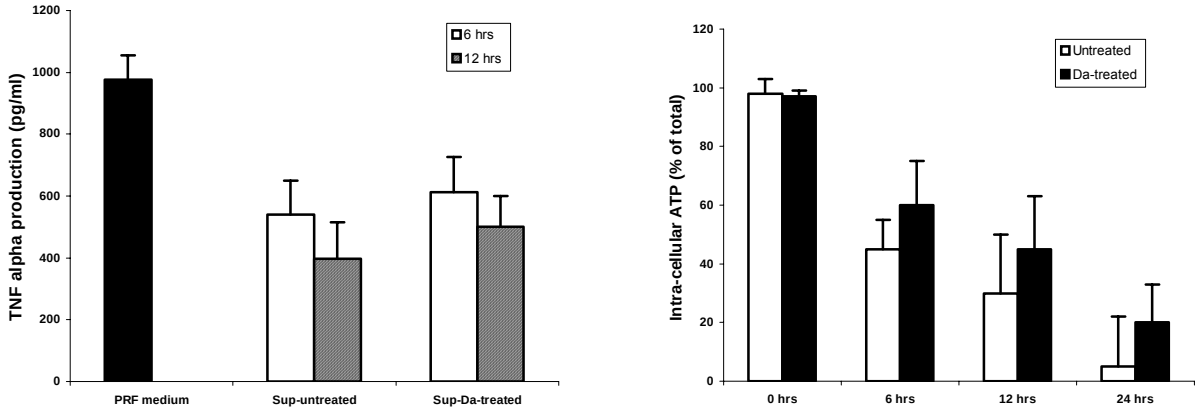


Figure 5 *Acquirement of TNFα suppressing activity in supernatants and intracellular ATP depletion in time.* ***A:*** *Whole blood was diluted 1:1 in normal PRF medium (filled bars) and in supernatants of untreated HUVEC (Sup-untreated) or in supernatants of dopamine treated HUVEC (Sup-Da-treated), obtained directly after 6 hrs (open bars) or 12 hrs (hatched bars) of cold storage. To each condition 0.5 µg/ml of LPS was added. TNFα production was assessed as described in figure 3. A total of 3 different experiments were performed. The result of a representative experiment is expressed as mean TNFα production (pg/ml) ± SD.* ***B:*** *Untreated (open bars) and dopamine treated (filled bars) HUVEC were stored at 4˚C for different periods of time. Hereafter, intra-cellular ATP was measured as described in materials and methods. The total amount of ATP before cold storage was taken as 100% for each condition. The result of a representative experiment is depicted and expressed as mean % of total ATP ± SD. A total number of three experiments were performed.*

Figure 6

A. **B.**

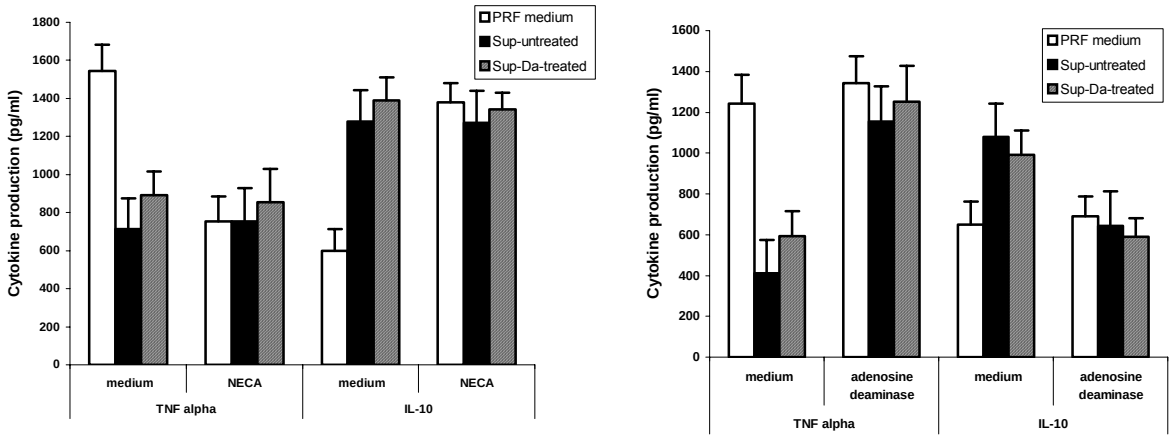


Figure 6 *Modulation of LPS induced cytokine production is mediated by adenosine.* ***A:*** *Whole blood was diluted 1:1 in normal PRF medium (open bars) and in supernatants of untreated HUVEC (filled bars; Sup-untreated) or in supernatants of dopamine treated HUVEC (hatched bars; Sup-Da-treated), obtained directly after 24 hrs of cold storage. To each condition 0.5 µg/ml of LPS was added. The whole blood assay was performed in the absence (medium) or presence of NECA (10µM)* ***B:*** *Similar as in* ***A,*** *but the supernatants were not treated (medium) or treated with adenosine deaminase (7U/ml) before initiation of the experiments. In* ***A*** *and* ***B****, TNFα and IL-10 production were assessed as described in figure 3. A total of 4 different experiments were performed. The result of a representative experiment is depicted and expressed as mean cytokine production (pg/ml) ± SD.*

Discussion

Long-term graft survival of renal allografts obtained from living donors is generally better than that obtained from post-mortem donors, despite better HLA-matching of the latter (32). According to the danger hypothesis (12), this might be explained by the fact that, immune activation following transplantation will be much stronger in allografts that have suffered from severe damage during the transplantation process. Compared to allografts from living donors, pre-transplantation injury in allografts from post-mortem donors is expected to be larger as a consequence of brain dead (33, 34) and prolonged hypothermic preservation (3). While brain death influences micro-circulation and organ perfusion (35) and is associated with inflammation in end-organs (33, 34), hypothermic preservation causes cell necrosis in a time dependent fashion (3, 8, 26). How hypothermic preservation might cause immune

activation is however not clear yet. In the present study we therefore investigated if hypothermic preservation of endothelial cells is associated with the release of HMGB1 and adenosine, two important molecules known to modulate innate immunity. The major findings of this study are first, HMGB1 expression is completely lost in endothelial cell that underwent necrosis during hypothermic preservation. This is not observed in endothelial cells rendered resistant to cold preservation damage by dopamine pre-treatment. Second, HMGB1 released in the supernatant of damage cells is biologically active as it upregulates adhesion molecules and IL-8 production in viable endothelial cells. Third, during hypothermic preservation adenosine is released independent of cell damage. Adenosine modulates TNFα and IL10 production in monocytes in an TLR4-dependent fashion.

To our knowledge, extra-nuclear HMGB1 expression in viable endothelial cells has thus far not been reported. Although Mullins et al (36) have reported that in LPS or TNFα stimulated HUVEC relocation of HMGB1 to the cytoplasm can occur, they did not mention relocation of HMGB1 to a specific intracellular compartment. Our findings on the extra-nuclear compartment of HMGB1 in HUVEC was unlikely due to a fixation or staining artefact, since HMGB1 expression in PBMC that were processed similarly as HUVEC, did not reveal an extra-nuclear HMGB1 compartment. Based on the HMGB1 staining pattern we speculate that this compartment might be related to the endoplasmic reticulum (ER), however this must be confirmed in future studies. In support of ER localization is a recent report from Ivanov et al (37) who showed co-localization of HMGB1 with markers of the ER, the ER Golgi intermediate compartment (ERGIC) and the Golgi in bone marrow derived dendritic cells and macrophages.

Since prolonged hypothermic preservation leads to tissue necrosis, the finding that HMGB1 was lost during cold preservation was not surprising. Cold preservation injury can be prevented by appropriate pre-treatment of cells with a number of compounds, e.g. catecholamines (8, 9) and iron scavengers (26, 38). Prevention of the loss of HMGB1 expression in dopamine treated HUVEC demonstrates that hypothermia *per se* does not cause the release of HMGB1 but that this reflects cell necrosis. Although, we did not address if HMGB1 expression was also reduced in organ allografts after hypothermic preservation, a number of studies have unambiguously demonstrated the occurrence of tissue necrosis under this condition (39, 25). Therefore it is likely that HMGB1 is indeed released during cold preservation of whole organs. The presence of HMGB1 in organ allografts might influence

neutrophil infiltration (40), endothelial production of pro-inflammatory cytokines (41), recruitment and proliferation of smooth muscle cells (42) and homing of endothelial progenitor cells (43).

Apart from HMGB1, adenosine could also be demonstrated in supernatants of HUVEC after cold storage. This might explain why supernatants of damaged cells downregulated TNFα production in whole blood assay, despite the presence of HMGB1. Adenosine release was associated with ATP hydrolysis and occurred independent of cell necrosis. Extra-cellular ATP can be converted to adenosine by a cascade of ectonucleotidases including CD39 and CD73, both of which are expressed on endothelial cells (44, 45). Because ATP hydrolysis also occurs intracellularly, adenosine can alternatively be released through nucleoside transporters (45).

We have previously *in vitro* demonstrated, that endothelial barrier dysfunction is restored after cold preservation by dopamine pre-treatment (9). Since adenosine enhances barrier function (44-47), this might underlie the beneficial effect of dopamine on barrier function. Although adenosine was also released during cold storage in untreated cells, it can not restore barrier dysfunction in these cells as they become necrotic with increasing cold preservation time (26). The involvement of adenosine on barrier function however, has not been directly addressed in the present study.

Because *in vitro* HMGB1 and adenosine are released in the supernatants and organ allografts are flushed before implantation, it can be argued that release of these factors do not have any significance in organ transplantation. Endothelial cells express the receptor for advanced glycation end products RAGE (48), a putative receptor for HMGB1, which might already bind its ligand during cold preservation. Subsequent warm reperfusion then can cause receptor activation leading to phenotypic and functional changes in endothelial cells. The relevance of endogenous release of adenosine during hypothermia is indeed questionable as adenosine is added to some preservation solution, e.g. UW.

In conclusion, our study identified two mediators, i.e. HMGB1 and adenosine, that are released during cold preservation and that are able to influence innate immunity. While the release of HMGB1 is associated with tissue necrosis and activation of innate immunity, adenosine release is independent of cell damage and suppresses activation of the innate immune system in an TLR4-dependent fashion. Prevention of cold preservation injury by

adequate donor treatment might thus prevent release of HMGB1 and preserve the action of adenosine on endothelial barrier function, thereby improving immediate graft function and long term graft survival after transplantation of organ allografts from post-mortem donors.

References

1. Maathuis MH, Leuvenink HJ, Ploeg RJ. Perspectives in organ preservation. *Transplantation* 2007; 83:1289-98
2. Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? *Cryobiology* 2007; 54:129-45
3. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney Int*. 2004; 65:713-8
4. Salahudeen AK. Consequences of cold ischemic injury of kidneys in clinical transplantation. *J Investig Med*. 2004; 52:296-8
5. Kieran NE, Rabb H. Immune responses in kidney preservation and reperfusion injury. *J Investig Med*. 2004; 52:310-4
6. Zieger MA, Gupta MP. Endothelial cell preservation at 10 degrees C minimizes catalytic iron, oxidative stress, and cold-induced injury. *Cell Transplant*. 2006; 15:499-510
7. Vairetti M, Griffini P, Pietrocola G, Richelmi P, Freitas I. Cold-induced apoptosis in isolated rat hepatocytes: protective role of glutathione. *Free Radic Biol Med* 2001; 31:954-61
8. Bartels-Stringer M, Kramers C, Wetzels JF, Russel FG, Groot H, Rauen U. Hypothermia causes a marked injury to rat proximal tubular cells that is aggravated by all currently used preservation solutions. *Cryobiology*. 2003; 47:82-91
9. Brinkkoetter PT, Beck GC, Gottmann U, et al. Hypothermia-induced loss of endothelial barrier function is restored after dopamine pre-treatment: role of p42/p44 activation. *Transplantation 2006*; 82:534-42
10. Verrier E. The microvascular cell and ischemia-reperfusion injury. *J Cardiovasc Pharmacol*. 1996; 27 Suppl 1:S26-30
11. Chandrasekar B, Smith JB, Freeman GL. Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. *Circulation*. 2001; 103:2296-302
12. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 1994; 12:991-1045
13. Mueller S, Scaffidi P, Degryse B et al, New EMBO member's review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J* 2001; 20:4337-40
14. Dumitriu IE, BarauH P, Manfredi AA, Bianchi ME, Rovere-Querini P. HMGB1: guiding immunity from within. *Trends Immunol*. 2005; 26:381-7
15. Popovic PJ, DeMarco L, Lotze MT et al. High mobility group B1 protein suppresses the human plasmacytoid dendritic cell response to TLR9 agonists. *J Immunol*. 2006; 177:8701-7
16. Scaffidi P, Misteli T, and Bianchi ME, Relaese of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418:191-5
17. Wang H, Bloom O, Zhang M, Ombrellino M, Che J, Vishnubhakat JM, Frazier A, Ivanova S, Borovikova L, Manogue K et al, HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285:248-51
18. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME and Rubartelli A, The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep*. 2002; 10:995-1001
19. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. *J Leukoc Biol*. 2007; 81:84-91
20. Harris HE, Raucci A. Alarming news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep*. 2006; 7:774-8
21. DeMarco RA, Fink MP, Lotze MP. Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1. *Mol Immunol*. 2005; 42:433-44
22. Adersson U, Wang H, Palmblad K, et al. High mobility group 1 protein (HMG-1] stimulates pro-inflammatory cytokine synthesis in human monocytes. *J. Exp. Med*. 2000; 192:565-70
23. Fiuza C, Bustin M, Talwar S et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003; 101:2652-60
24. Park JS, Svetkauskaite D, He QB et al. Involvement of Toll Like Receptor 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol. Chem*. 2004; 279:7370-77
25. Huet PM, Nagaoka MR, Desbiens G et al. Sinusoidal endothelial cell and hepatocyte death following cold ischemia-warm reperfusion of the rat liver. *Hepatology* 2004; 39:1110-19
26. Yard B, Beck G, Schnuelle P, et al. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. *Am J Transplant*. 2004; 4:22-30
27. Gujral JS, Bucci TJ, Farhood A and Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? *Hepatology* 2001; 33:397-405
28. Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. J Appl Physiol 1994; 76:5-13,
29. Van Belle H, F Goossens, and J Wynants. Formation and release of purine catabolites during hypoperfusion, anoxia and ischemia. *Am. J. Physiol*. 1987; 252:H886-93

30. Matherne GP, JP Headrick, SD Coleman, and RM Berne. Interstitial transudate purines in normoxic and hypoxic immature and mature rabbit hearts. *Pediatr. Res*. 1990; 28:348-53
31. Morris GE, Parker LC, Ward JR, et al. Cooperative molecular and cellular networks regulate Toll-like receptor-dependent inflammatory responses. *FASEB* 2006; 20: 1539-1549
32. Terasaki PI,Cecka JM, Gjetson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. *N Engl J Med*. 1995; 333:333-6
33. Weiss S, Kotsch K, Francuski M, et al. Brain death activates donor organs and is associated with a worse I/R injury after liver transplantation. *Am J Transplant*. 2007; 7:1584-93
34. Pratscke J, Wilhelm MJ, Laskowski I, et al. Influence of donor brain death on chronic rejection of renal transplants in rats. *J Am Soc Nephrol*. 2001; 12:2474-81
35. Herijgers P, Leunens V, Tjandra-Maga TB, Mubagwa K, Flameng W. Changes in organ perfusion after brain death in the rat and its relation to circulating catecholamines. *Transplantation*. 1996; 62:330-5
36. Mullins GE, Suden-Gullberg J, Johansson AS, et al. Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. *Scand J Immunol*. 2004; 60:566-73
37. Ivanov S, Dragoi A-M, Wang X, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood*. 2007; 110:1970-81
38. Rauen U, Kerkweg U, de Groot H. Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions. *Cryobiology*. 2007; 54:77-86
39. Reddy SP, Battacharjya S, Maniakin N, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. *Transplantation*. 2004; 77:1328-32
40. Orlova VV, Choi EY, Xie C, et al. A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. *EMBO J*. 2007; 26:1129-39
41. Fiuza C, Bustin M, Talwar S, et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood*. 2003; 101:2652-60
42. Porto A, Palumbo R, Pieroni M, et al. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. *FASEB J*. 2006; 20:2565-6
43. Chavakis E, Hain A, Vincini M, et al. High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells. *Circ Res*. 2007; 100:204-12.
44. Eltzschig HK, Iba JC, Furuta GT, et al. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. *J Exp Med*. 2003; 198:783-96
45. Eltzschig HK, Weissmuller T, Mager A, Eckle T. Nucleotide metabolism and cell-cell interactions. *Methods Mol Biol*. 2006; 341:73-87
46. Satpathy M, Gallagher P, Jin Y, Srinivas SP. Extracellular ATP opposes thrombin-induced myosin light chain phosphorylation and loss of barrier integrity in corneal endothelial cells. *Exp Eye Res*. 2005; 81:183-92
47. Eltzschig HK, Eckle T, Mager A, et al. ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. *Circ Res*. 2006; 99:1100-8
48. Yonekura H, Yamamoto Y, Sakurai S, Watanabe T, Yamamoto H. Roles of the receptor for advanced glycation endproducts in diabetes-induced vascular injury. *J Pharmacol Sci.* 2005; 97:305-11

Chapter 7

The anti-inflammatory effect of CO releasing molecules is not perpetuated by induction of heme oxygenase 1

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Abstract

Heme oxygenase-1 (HO-1) exerts anti-inflammatory effects via heme degradation, resulting in the generation of biliverdin, Fe^{2+} and carbon monoxide (CO). Since CO itself up-regulates HO-1 expression, perpetuation of the anti-inflammatory effect is expected. We investigated how CO releasing molecules (CORM), i.e. CORM-3, modulate the expression of adhesion molecules on endothelial cells and if HO-1 mediated perpetuation was involved.

CORM-3 consistently inhibited the expression of VCAM-1 and E-selectin on TNF-α stimulated human umbilical vein endothelial cells (HUVEC), while ICAM-1 expression was marginally affected and only in 3 out of 8 lines tested. Although CORM-3 did not influence initial NFκB-activation, this was significantly reduced at later time-points. Down-regulation of VCAM-1 and E-selectin expression by CORM-3 also occurred when CORM-3 was added 24 hrs after TNF-α stimulation or when TNF-α was removed. This was paralleled by deactivation of NFκB, concomitantly with a reduction in VCAM-1 mRNA. Although TNF-α removal was more effective in this regard, VCAM protein was down-regulated more rapidly when CORM-3 was added compared to TNF-α removal. Induction of HO-1 occurred in a dose- and time-dependent manner and was mediated by Nrf2. Neither in HO-1 nor in Nrf2 siRNA-transfected HUVEC the efficacy of CORM-3 to down-regulate VCAM-1 expression was lost. In Nrf2 siRNA-transfected HUVEC CORM-3 no longer induced HO-1.

In conclusion, our study demonstrates that CORM-3-mediated anti-inflammatory effects are independent of HO-1 up-regulation. Although CORM-3 most likely exerts its effect by inhibition of sustained NFκB activation, down-regulation of VCAM expression also seems to be regulated post-translationally.

Introduction

Leukocyte extravasation occurring at the onset of inflammation is a highly regulated process characterized by bidirectional communication between endothelial cells and leukocytes. Upon exposure of endothelial cells to inflammatory mediators, adhesion molecules and chemokines are rapidly upregulated, which in turn facilitate leukocyte migration (1-4). In addition, ligand binding to CXC chemokine receptor 1 (CXCR1) and CXCR2 stimulates neutrophils to release a number of factors including proteases, cytokines, chemokines, and other chemoattractants that amplify inflammation and extend duration of the latter (5). Hence, understanding the mechanisms controlling down-regulation of pro-inflammatory cytokines and adhesion molecules is now being widely recognized as a prerequisite for the identification of novel drug targets for inflammatory disease (2, 6, 7).

In addition to its unambiguous role in oxidant-induced injury (8-10), unequivocal evidence demonstrates that the heme oxygenase system (HO) is involved in the control of inflammatory processes (11-13, 16). The HO system comprises several isoenzymes (14, 15), of which the inducible HO-1 isoenzyme appears to be particularly important as anti-inflammatory mediator (11-13, 17). HOs are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe^{2+} and biliverdin, the latter being subsequently converted to bilirubin (18, 19).

Although a number of studies have postulated putative mechanisms by which HO-1 exerts its anti-inflammatory effect, some of the published data are controversial. While Soares et al have shown that HO-1down-regulates VCAM and E-selectin expression via bilirubin and iron chelation with no apparent involvement of CO (12, 13), Otterbein et al and Sethi et al clearly demonstrate the anti-inflammatory potential of CO in macrophages and monocytes (11) as well as in endothelial cells (20, 21). The salutary effect of CO has also been shown for organ transplantation and ischemia reperfusion injury (22, 23).

Recently, a new class of molecules, termed CO-releasing molecules (CORM), has been described (24) that are composed of transition metal carbonyls. They are capable of liberating CO under appropriate conditions. In particular, CORM-3 (tricarbonylchloro(glyconato)ruthenium(II)) and CORM-A1 (sodium boranocarbonate), which both are fully water-soluble, rapidly liberate CO when dissolved in physiological solutions. These molecules might therefore be of therapeutically interest to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion (25). In addition, these

molecules have been widely used to increase our understanding of the biological function of CO (26-27).

Interestingly, CO itself can induce the expression of HO-1 in an Nrf2-dependent fashion as was demonstrated in hepatoma cells (29). In addition, CO stabilizes HIF-1α, a putative transcription factor for HO-1 expression, in macrophages (30). Since induction of HO-1 by CO might perpetuate the anti-inflammatory effect of CO we now investigated whether (A) HO-1 is induced by CORM-3 in endothelial cells, (B) its expression is regulated by the transcription factors Nrf2 or HIF-1α, and (C) if the anti-inflammatory effect of CORM was perpetuated by HO-1.

Materials and methods

Reagents

Reagents were obtained from the sources as indicated: endothelial cell culture medium (Promocell, Heidelberg, Germany), PBS (GIBCO, invitrogen, NY), fetal bovine serum (FBS) Gold (PAA laboratories GmbH, Pasching, Austria), trypsin/EDTA solution, DMSO, Tween 20, DEAE-Dextran, chloroquine, Hepes, Triton X-100, DTT, sodium deoxycholate, Tris-base, EDTA, APS, SDS, TEMED, tricarbonyldichlororuthenium(II) dimer, glycine, sodium ethoxide, human recombinant TNF-α (Sigma, St. Louis, MO), bovine serum albumin (SERVA, Heidelberg, Germany), protease inhibitor cocktail, 1st strand cDNA synthesis Kit (Roche Diagnostic, Mannheim, Germany), NF-κB consensus oligonucleotides, Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany), Coomassie protein assay reagent (Pierce, Rockford, IL), Trizol (Invitrogen, Carlsbad, CA), chloroform, isopropanol, tetrahydrofuran, β-mercaptoethanol (Merck, Darmstadt, Germany). Primers and all reagents were purchased for TaqMan PCR (ABI, Darmstadt, Germany). All antibodies for flow cytometric analysis were purchased from R&D System (Minneapolis, MN) and all FACS reagents from Becton Dickinson (Heidelberg, Germany). All antibodies used for Western blotting, including horseradish peroxidase (HRP) conjugates, antibodies for supershifts as well as siRNAs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) with exception of the anti-HO-1 (Stressgen, Victoria, Canada) and the anti-GAPDH antibodies (Abcam Cambridge, UK). chemiluminescence reagent was purchased from PerkinElmer LAS Inc. (Boston, MA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords as described previously (31). The cells were grown in basal endothelial medium supplemented with 10% FBS and essential growth factors until they formed a confluent monolayer. Cells were stimulated with 50ng/ml of TNF-α in the presence or absence of different concentrations of CORM-3. Unstimulated cells served as control.

Synthesis of tricarbonylchloro(glycinato)ruthenium(II)

Tricarbonylchloro(glycinato)ruthenium(II) ([$Ru(CO)_3Cl$(glycinate)]) was synthesized starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$). Briefly, $[Ru(CO)_3Cl_2]_2$ (0.5 g) and glycine (0.151 g) were placed under nitrogen in a round-bottomed flask. Methanol (291 ml) and sodium ethoxide (0.132 g) were added and the reaction was allowed to continue under stirring for 18 hrs at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran (THF). The yellow solution was evaporated down to give a pale yellow solid (yield 92-96%) and was stored in closed vials at -20ºC. For each experiment CORM-3 was dissolved freshly in PBS.

FACS analysis

HUVECs were cultured in medium supplemented with 50ng/ml TNF-α in the presence or absence of 1mM CORM-3. HUVECs cultured in endothelial medium were used as control. FACS analysis was performed with 2×10^6 cells using the following FITC-conjugated monoclonal antibodies: anti-human ICAM-1 (BBIG-I1), anti-human VCAM-1 (BBIG-V3) and anti-human E-selectin (BBIG-E5). Antibodies were added for 40 min at 4ºC followed by extensive washing with PBS. FACS analysis was performed on a FACScalibur equipped with the CELLQuest software. The data were analysed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

Westernblot analysis

HUVECs were stimulated for different time periods with 50ng/ml TNF-α in the presence or absence of 1mM CORM. Hereafter, the cells were harvested and lysed in 50 µl lysis buffer containing 10mM Tris-base, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1µM DTT and proteinase inhibitor cocktail. In some experiments, nuclear proteins were isolated as previously described (32, 33). Protein concentrations were assessed using Coommassie reagents. SDS-PAGE and Western blotting were performed essentially

similar as described (31). Briefly, samples were resolved on 10 % SDS-PAGE and transferred onto PVDF filters by semi-dry blotting. The filters were incubated with 5% non-fat dry milk powder in PBS for 1 hour at room temperature to block unspecific background staining and hereafter incubated overnight at 4^{o}C with specific polyclonal antibodies, depending on the experiment that was performed. The following antibodies were used: polyclonal rabbit anti-HO-1 antibody, polyclonal rabbit anti-Nrf2 antibody, polyclonal rabbit anti-IκB alpha antibody, polyclonal goat anti-VCAM-1 antibody, polyclonal rabbit anti-NF-κB p50 antibody, polyclonal rabbit anti- NF-κB p65 antibody. After extensive washing in PBS/Tween/5 % non fat dry milk powder, the filters were incubated 60 minutes with appropriate horseradish peroxidase-conjugated polyclonal IgG, followed by 3 times washing in PBS/Tween. Visualization of immunoreactive bands was performed by chemiluminescence reagent according to the manufacturer's instructions. The filters were re-probed with monoclonal anti-Histone H1 antibody or monoclonal anti-GAPDH antibody to demonstrate equal loading.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from HUVECs as described above. Protein concentrations were determined by Bradford assay. EMSA was performed essentially as previously described (32, 33). Briefly, NF-κB consensus oligonucleotides were labelled to a specific activity >5x10^{7}cpm/µg DNA. 10 µg of nuclear extracts were added to 1 ng of labelled oligonucleotide in a total volume of 20 µl containing 10 mM HEPES (pH=7,5), 0.5 mM EDTA, 70 mM KCl, 2 mM DTT, 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 M PMSF, 1 mg/ml BSA and 0.1 µg/µl poly di/dc. In each experiment specificity of binding was demonstrated by adding cold consensus or mutated NF-κB oligonucluotides to the nuclear extracts. In addition, supershifts were performed by adding anti-p65 and p55 antibodies to the samples. DNA-protein complexes were separated on 5% non-denaturating polyacrylamide gels electrophoresed in low ionic strength buffer and visualized by autoradiography.

RNA isolation, reverse transcriptase PCR, quantitative TaqMan PCR

Total RNA was isolated from confluent endothelial cell monolayers using Trizol®-Reagent. Thereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Darmstadt, Germany). RNA concentration and quality were assessed by RNA 6000 Nano assays on a Bioanalyzer 2100 system (Agilent, Boeblingen, Germany). 500 ng of total RNA was reverse-

transcribed into cDNA using the 1st Strand cDNA Synthesis Kit. cDNA was diluted in 20 µl DEPC-treated water and stored at –20°C until use.

For reverse transcriptase PCR 1µl of cDNA was amplified in a 50µl reaction mix containing 10mM dNTPs, 50pM of each primer, 2.5 units Taq polymerase and 1.5mM $MgCl_2$. The primers used were as follows: HO-1 forward: 5'- GCT CAA CAT CCA GCT CTT TGA GG-3' and reverse: 5'- GAC AAA GTT CAT GGC CCT GGG A-3'; VCAM forward: 5'- CGA TCA CAG TCA AGT GTT CAG TTG-3' and reserve: 5'- GCA ATT CTT TTA CAG CCT GCC T-3'; GAPDH forward: 5'- GTC TTC ACC ACC ATG GAG AA-3' and reserve: 5'- ATC CAC AGT CTT CTG GGT GG-3'. The cycling conditions used for various primers were as follows: 4 min of denaturation at 94°C, followed by 28 (VCAM-1) or 25 (HO-1 and GAPDH) cycles of amplification, each consisting of denaturation for 30s at 94°C, annealing for 30s at 59°C (VCAM-1), 62 °C (HO-1) or 55°C (GAPDH) and extension for 45s at 72°C. After the last amplification a final extension for 10 min at 72°C was performed for each reaction. PCR products were analysed on a 1 % agarose gel containing ethidium bromide.

Quantitative real-time RT-PCR was performed on the ABI-Prism 7700 sequence detection system with the TaqMan universal PCR master mix No AmpErase UNG (part no. 4324018). Taqman probes for VCAM-1 (part No. HS00174239_m1) and β-actin (part No. HS99999903_m1). Samples were run under the following conditions: initial denaturation for 10 minutes at 95° C followed by 40 cycles of 15 s at 95° C and 1 minute at 60° C. The levels of gene expression in each sample were determined with the comparative cycle threshold method. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90% and 100%. Linearity of the assay could be demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of β-actin. Each experiment was repeated 3 times with similar results.

Reporter assays

HUVECs were transfected with a reporter construct containing 3 hypoxia response elements (3HRE.luc) (34) or a construct containing an anti-oxidant response element from the HO-1 promoter (HO-ARE.luc) (35). All transfections were performed using DEAE-dextran as described previously (36) and in each case the ubiquitin-dependent Renilla luciferase reporter was co-transfected. Two days after transfection, cells were stimulated for 24 hrs with CORM-3. Luciferase activity was measured using the Dual-Glo Luciferase Assay System. All experiments were performed in triplicate and ARE- or HRE-dependent luciferase activities

were normalized for luciferase acivity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared to unstimulated controls.

Cell transfection with siRNA

HUVECs were seeded in 12-well plate at a density of 0.5-2×10^5 one day before transfection with HO-1 siRNA, Nrf2 siRNA or control siRNA. Transfection was performed according to the manufacturer's instructions. Briefly, cells were incubated for 6 hrs in transfection medium supplemented with siRNA and transfection reagent. Hereafter, endothelial cell culture medium containing 20% FBS was added without removing the transfection solution and the cells were allowed to grow for an additional 24hrs. For each experiment the efficacy of siRNA was demonstrated by disappearance of the specific band in Western blot analysis.

Statistical analysis

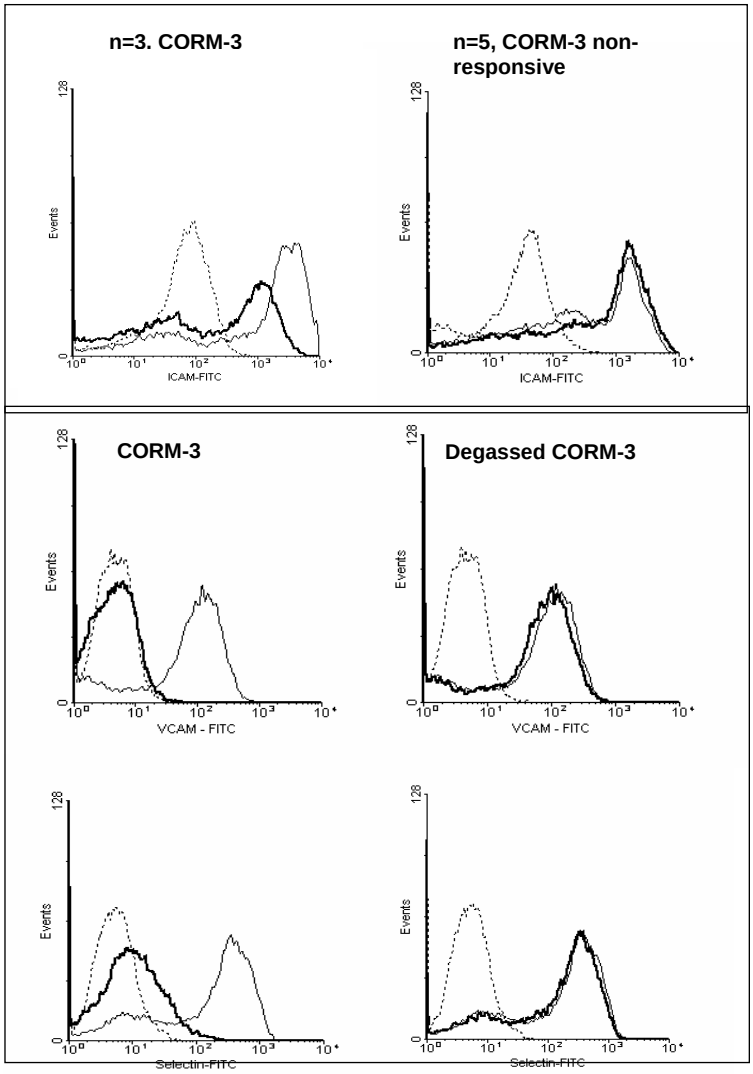
Data are presented as mean ± SD for the indicated number of separate experiments. All analyses were based on more than three separate experiments. Differences between groups were determined by Student's *t* test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Inhibition of adhesion molecules by CORM

CORM-3 inhibited TNF-α-mediated induction of VCAM-1 and E-selectin in all cell lines tested (n=8), while inhibition of ICAM-1 only occurred in 3 of these (figure 1A). Inhibition of adhesion molecule expression was mediated by the release of CO since a degassed solution of CORM-3 was ineffective (figure 1A, lower panel). To exclude that loss of adhesion molecule expression was due to proteolytic cleavage from the cell membrane, Western blot analysis with whole cell lysates was performed. As demonstrated for VCAM-1, induction by TNF-α was significantly attenuated by CORM-3 and was completely absent when endothelial cells were stimulated for 24 hrs in the presence of CORM-3 (figure 1B).

Figure 1 **A**



B

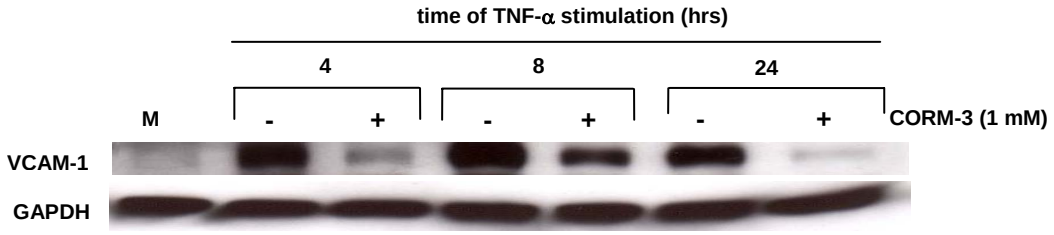


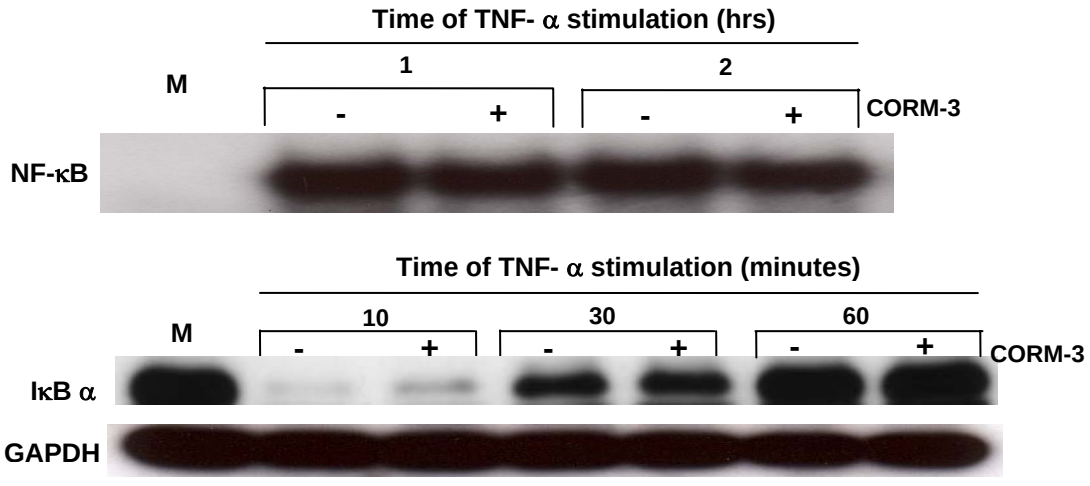
Figure 1 *Modulation of TNF-α induced expression of adhesion molecules by CORM-3.* ***(A):*** *Eight different HUVEC lines were stimulated for 24 hrs with 50 ng/ml of TNF-α in the absence (normal line) or presence of 1 mM of CORM-3 (bold line) and surface expression of ICAM-1, VCAM-1 and E-selectin determined by flow cytometry. Basal expression of adhesion molecules is also depicted (dotted line). While limited TNF-a-induced expression of ICAM-1 occurred only in 3 out of 8 cell lines exposed to CORM-3 (upper panel), a weaker induction of VCAM-1 and E-selectin by TNF-a was consistently observed. Degassed CORM-3 solution revealed no effect (lower right panel).* ***(B):*** *Time-dependent modulation of VCAM-1 expression by CORM-3. HUVEC were stimulated for different time periods with 50 ng/ml of TNF-α in the absence (-) or presence of 1 mM of CORM-3 (+). Hereafter, cell lysates were*

prepared and subjected to Western blotting. Membranes were incubated with anti-VCAM-1 antibody and hereafter reprobed with anti-GAPDH antibody to control for equal loading. A total of 3 independent experiments were performed, the result of a representative experiment is shown.

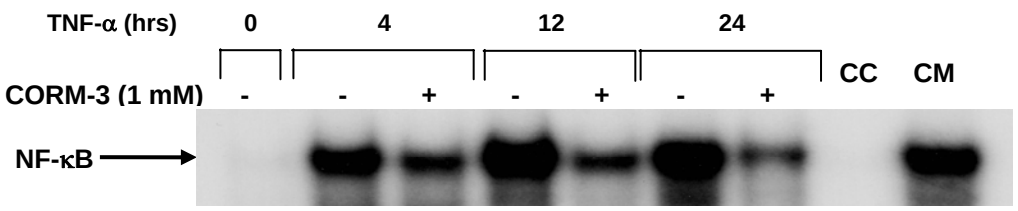
CORM-3 acts through the NF-κB pathway

Since up-regulation of the adhesion molecules studied depends on activation of NF-κB, we next assessed if CORM-3 interferes with this process. Within one hour of TNF-α stimulation NF-κB-binding activity was detected in nuclear extracts of endothelial cells. However, neither at this time point nor after 2 hrs of TNF-α stimulation CORM-3 significantly influenced NF-κB binding activity (figure 2A upper panel). In line with this, no influence of CORM-3 on the degradation of IκBα was observed (figure 2A, lower panel). In contrast to these early time points, NF-κB binding activity was significantly reduced after 4 hrs of TNF-α stimulation in CORM-3-treated cells and gradually declined (figure 2B). This was reflected by a decrease in the nuclear expression of NF-κB p65. Nuclear expression of NF-κB p50, however, was not changed by CORM-3 (figure 2C).

Figure 2 A



B



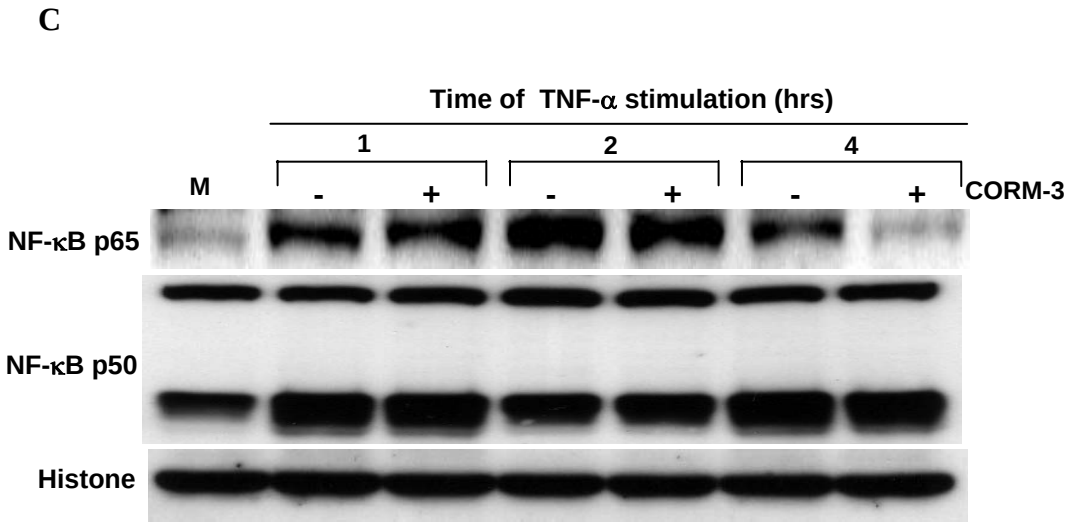


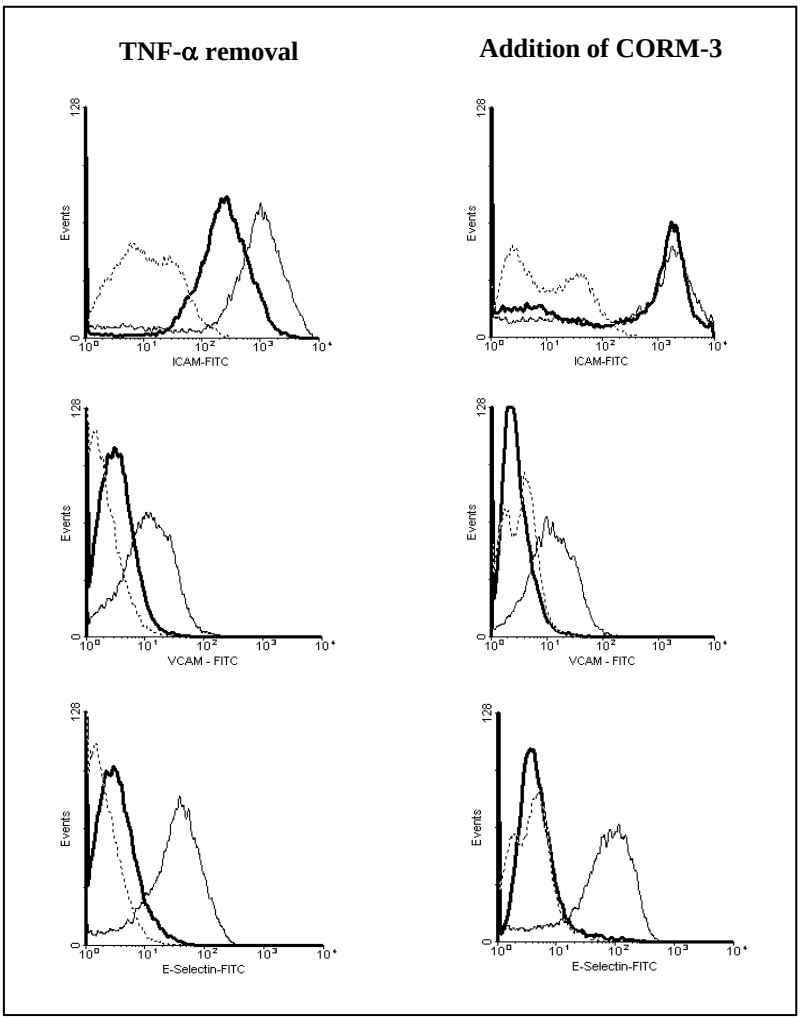
Figure 2 Influence of CORM-3 on TNF-α-mediated NF-κB activation. ***(A):*** *Endothelial cells were stimulated for 1 or 2 hrs with 50 ng/ml of TNF-α in the absence (-) or presence of 1 mM of CORM-3 (+). Endothelial cells exposed to culture medium (M) only served as control. Nuclear extracts were prepared and used for EMSA as described in Materials and Methods (upper panel). To assess the influence of CORM-3 on IκBα degradation, cells were stimulated for different time periods using the same concentrations of TNF-α and CORM-3. Cell lysates were analysed by Western blot for IκBα and GAPDH to demonstrate equal sample loading (lower panel).* ***(B):*** *Influence of CORM-3 on NF-κB activation was also analysed at later time points. HUVEC were stimulated as in (A) and nuclear extracts were prepared at 0, 4, 12 and 24 hrs after TNF-α stimulation. Specificity of the shifted bands was demonstrated by addition of an excess of cold consensus (CC) or cold mutant (CM) oligonucleotides to the samples.* ***(C):*** *Nuclear expression of NF-κB p65 and NF-κB p50 in TNF-α-stimulated HUVEC. Cells were exposed to TNF-α as above in the presence (+) or absence (-) of 1 mM of CORM-3. At the time intervals indicated nuclear extracts were obtained and studied for expression of p65 and p50. To confirm equal loading of lanes, membranes were stained with anti-histone antibodies. In A, B and C, the results of 1 out of 4 individual experiments are shown.*

The presence of TNF-α was required to maintain VCAM-1 and E-selectin expression on endothelial cells, since an almost complete down-regulation of these adhesion molecules occurred within 24 hrs after TNF-α removal. Although the expression of ICAM was also down-regulated when TNF-α was removed from the culture medium, it was still increased when compared to basal expression levels. Interestingly, when CORM-3 was added after 24 hrs to the TNF-α-containing culture medium, this also resulted in a complete loss of VCAM-1 and E-selectin expression (figure 3A). In contrast, the expression of ICAM-1 was not influenced. Hence, CORM-3 is able to down-regulate VCAM-1and E-selectin even in the continued presence of TNF-α.

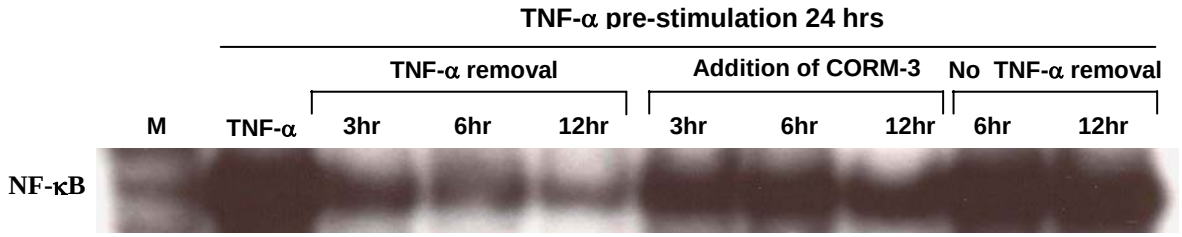
While TNF-α removal resulted in a rapid decrease in nuclear NF-κB binding activity evident already 3 hrs after removal, NF-κB binding activity was attenuated at early time-points after CORM-3 addition. Twelve hrs after addition of CORM-3, however, NF-κB binding activity was also decreased in these cells (figure 3B). In line with these observations, steady-state VCAM-1 mRNA expression decreased much faster when TNF-α was removed compared to addition of CORM-3 (figure 3C). Nevertheless, in time response experiments we repeatedly observed that disappearance of VCAM-1 protein occurred much faster upon addition of CORM-3 than after TNF-α removal (figure 3D). CORM-3 did not influence stabilization of VCAM-1 mRNA since steady-state VCAM-1 mRNA decreased to a similar extent in actinomycin D-treated HUVEC both in the absence or presence of CORM-3 (data not shown).

Figure 3

A



B



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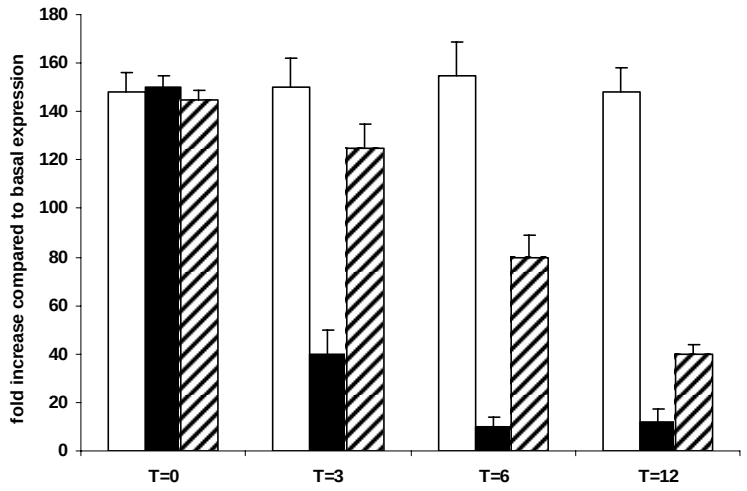

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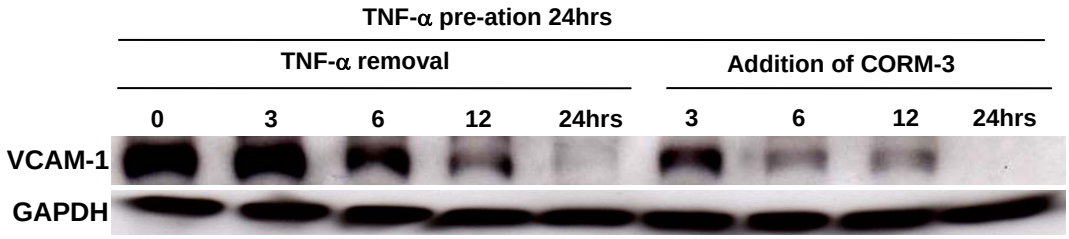


Figure 3 *Influence of CORM-3 or TNF-α removal on (A) surface expression of adhesion molecules, (B) NF-κB activation, (C) expression of VCAM-1 mRNA and (D) expression of VCAM-1 protein. HUVEC were stimulated for 24 hrs with 50 ng/ml of TNF-α. Hereafter, the cells were washed to remove TNF-α or CORM-3 was added in the continued presence of TNF-α. Cells that were kept in the presence of TNF-α alone for similar time periods served as control.* ***(A):*** *Surface expression of ICAM-1, VCAM-1 and E-selectin after TNF-α stimulation (normal line), after addition of CORM-3 or removal of TNF-α (bold line). Basal expression of these adhesion molecules is also depicted (dotted line).* ***(B):*** *Nuclear extracts were prepared for electrophoretic mobility shift assays at 3, 6 and 12 hrs after TNF-α removal or after addition of CORM-3. Nuclear extracts were also prepared directly after 24 hrs of TNF-α stimulation (TNF-α) or from cells that were kept in the presence of TNF-α but not exposed to CORM-3 (no TNF-α removal). Cells that were kept in culture medium (M)*

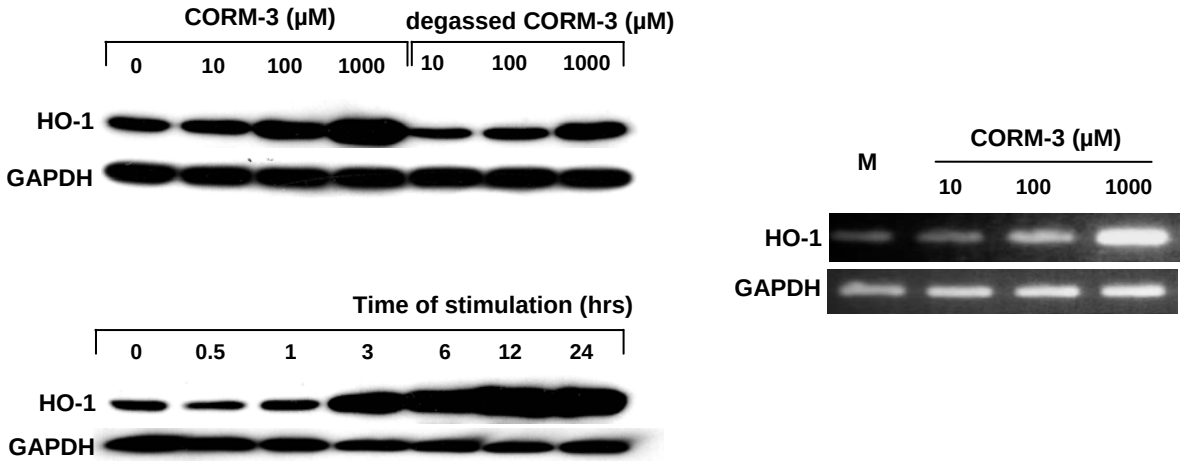
were included to demonstrate TNF-α-mediated NF-κB activation. ***(C):*** *Steady-state VCAM-1 mRNA expression. HUVEC were stimulated with TNF-α for 24 hrs. Hereafter, cells were kept in the presence of TNF-α (open bars) or washed (filled bars) or CORM-3 was added to the cells (hatched bars). Subsequently, the cells were cultured for various time periods and total RNA was isolated. Results are expressed as fold increase compared to basal VCAM-1 mRNA expression. Significant differences were found between TNF-α removal and CORM-3 addition at T=3 hrs, T=6 hrs ($p<0.01$ for both time points) and T=12 hrs ($p<0.05$).* ***(D):*** *HUVEC were treated as in C. At various time periods after TNF-α removal or CORM-3 addition, cell extracts were prepared. VCAM-1 protein was analysed by Western blotting. In A, B, C and D the results of representative experiments are shown. At least 3 different experiments were performed.*

HO-1 is induced by CORM-3 in an Nrf2-dependent fashion

CORM-3 induced the expression of HO-1 in a time- and dose-dependent manner (figure 4A). While HO-1 was already slightly upregulated at a concentration of 100 μM, this was much more pronounced when 1 mM was used (figure 4A, upper panel). At the latter concentration upregulation of HO-1 protein was apparent already 3 hrs after addition of CORM-3 (figure 4A, middle panel). Degassing of the CORM containing solution did not completely abrogate upregulation of HO-1, suggesting that this was partly mediated by the tricarbonylchlororuthenium(II) derivative itself (figure 4A, upper panel). PCR analysis revealed that upregulation of HO-1 mRNA occurred at similar concentrations as was observed for HO-1 protein (figure 4A, right panel).

Figure 4

A



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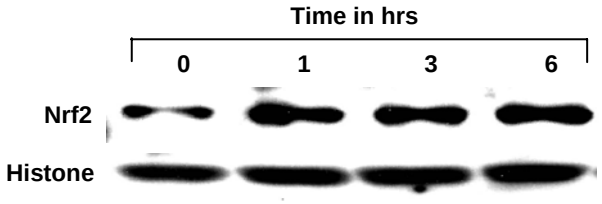

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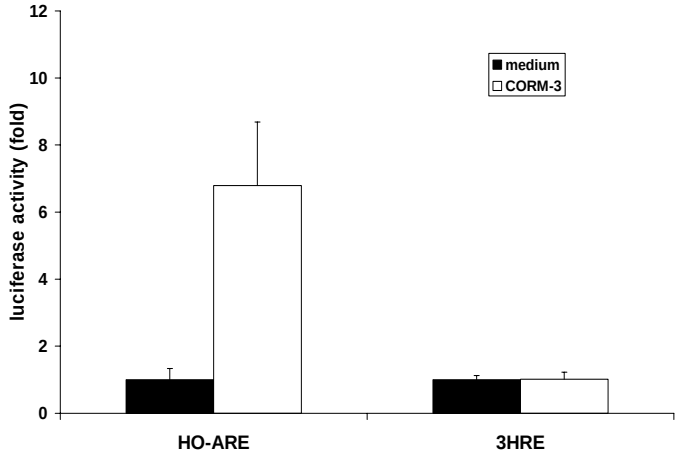


Figure 4 *Induction of HO-1 by CORM-3.* ***(A):*** *HUVEC were incubated for 24 hrs with different concentrations of CORM-3 or degassed CORM-3. Hereafter, cell lysates were studied by Western blot analysis for HO-1 and GAPDH expression (upper panel). In a second set of experiments, HUVEC were incubated for different time periods with 1 mM of CORM-3. The expression of HO-1 was assessed by Western blotting (middle panel). To demonstrate that HO-1 expression was regulated at the transcriptional level, total RNA was isolated from HUVEC that were incubated with different concentrations of CORM-3. RT-PCR for HO-1 and GAPDH was subsequently performed (right panel).* ***(B):*** *Nuclear translocation of Nrf2 was assessed by Western blot analysis of nuclear protein isolated from HUVEC that were stimulated for different time periods with 1 mM of CORM-3.* ***(C):*** *HUVEC were transfected with reporter constructs containing either the ARE consensus sequence of the HO-1 promoter (HO-ARE) or with a reporter construct containing 3 hypoxia responsive elements (3HRE). In each case co-transfection was performed with the ubiquitin-dependent Renilla luciferase reporter. Luciferase activities were normalized for luciferase acivity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared to unstimulated controls. Significance was only found for the HO-ARE reporter, $p<0.01$ untreated vs. CORM-3 treated. In A, B and C the results of representative experiments are shown. At least 4 different experiments were performed.*

Since these data suggested that CORM-3-mediated HO-1 expression was transcriptionally regulated, we next investigated the involvement of three transcription factors that have been shown to influence HO-1 transcription. Neither NF-κB activation nor HIF-1α accumulation

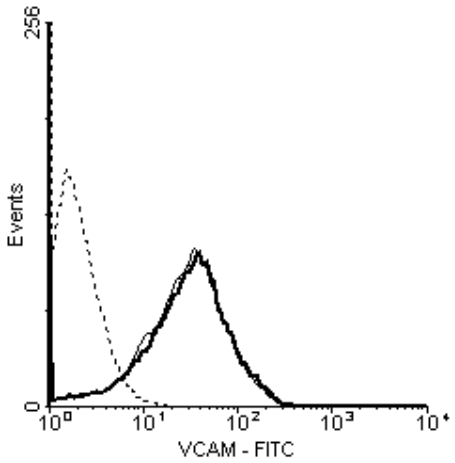
occurred after CORM treatment (data not shown). In contrast, nuclear translocation of Nrf2 was clearly evident 3 hrs after CORM-3 treatment (figure 4B). CORM-3 did not induce expression of a hypoxia-responsive element-dependent luciferase reporter construct, while this was the case in cells transfected with an Nrf2 reporter (figure 4C).

CORM-3 induced HO-1 expression does not contribute to inhibition of VCAM-1 expression

Since CORM-3 induces HO-1 expression, this might further increase intracellular CO concentrations and hence may perpetuate down-regulation of adhesion molecules in the presence of TNF-α. To address this issue, we first stimulated endothelial cells with 1 mM of CORM-3 to induce HO-1 followed by a challenge with TNF-α. Despite an increase in HO-1 expression, induction of VCAM-1 by TNF-α was not influenced in these cells (figure 5A). To formally exclude a role for HO-1 in down regulation of VCAM-1 expression we also used a siRNA approach to deplete HO-1 or Nrf2. The expression of both HO-1 and Nrf2 was almost completely blocked by this approach (figure 5B). Nevertheless, neither TNF-α-induced VCAM-1 expression, nor inhibition hereof by CORM-3 was significantly changed in HO-1- and Nrf2-depleted endothelial cells, respectively (figure 5C upper panel). Compatible with the pivotal role of Nrf2 in HO-1 induction, Nrf2 siRNA treatment abolished the upregulation of HO-1 by CORM-3 (figure 5C, lower panel).

Figure 5

A



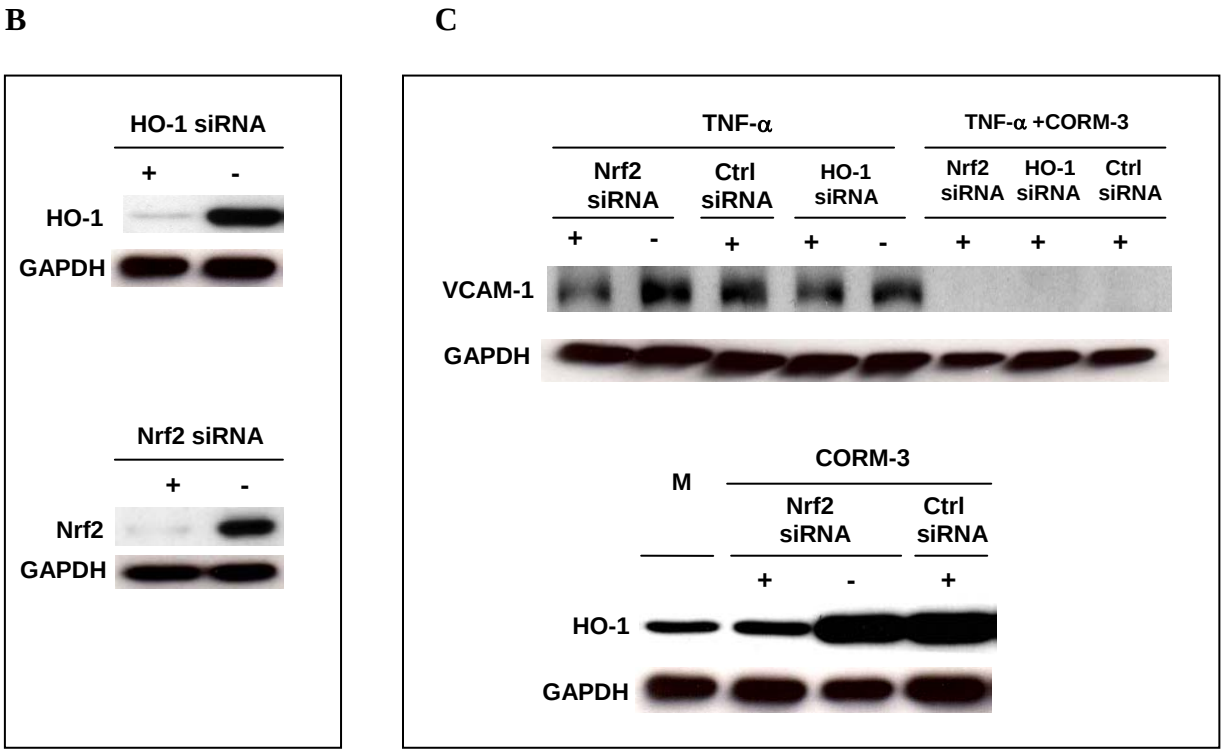


Figure 5 *Induction of HO-1 is not involved in CORM-3 mediated downregulation of VCAM-1 expression.* ***(A):*** *HUVEC were incubated for 24 hrs with CORM-3 (bold line) or left untreated (normal line). Hereafter, the cells were washed and stimulated for 24 hrs with 50 ng/ml of TNF-α. Basal expression of VCAM-1 is depicted as dotted histogram.* ***(B):*** *HUVEC were transfected with Nrf2, HO-1 or control siRNA. Two days after transfection the expression of HO-1 and Nrf2 was assessed by Western blot analysis.* ***(C):*** *Transfected cells were subsequently stimulated for 24 hrs with TNF-α (50ng/ml) or TNF-α plus CORM-3 (1 mM). The expression of VCAM-1 (upper panel) and HO-1 (lower panel) was assessed by Western blotting. The results of representative experiments are shown. At least 3 different experiments were performed.*

Discussion

Based on current evidence, the HO-1 pathway is of undisputable importance for the control of inflammation. Overexpression of HO-1 modulates pro-inflammatory processes in a number of cells including macrophages and endothelial cells (11, 12, 37, 38), yet, the mechanisms by which HO-1 exerts its salutary effects are not completely delineated. In the present study we investigated the role of HO-1 in perpetuation of the anti-inflammatory effect of CORM-3. The main findings of this study were the following. First, CORM-3 consistently inhibited the upregulation of VCAM-1 and E-selectin after TNF-α stimulation, but the influence on ICAM-1 was only marginal. Second, initial activation of the NF-κB pathway by TNF-α is not influenced by CORM-3. Instead, CORM-3 deactivates sustained activation of this pathway.

Third, HO-1 is induced by CORM-3 in an Nrf2 dependent fashion. Fourth, induction of HO-1 does not contribute to down-regulation of VCAM-1 and E-selectin expression by CORM-3.

There is some controversy on the main mediators through which HO-1 acts as a modulator of inflammation. Clearly, this might be related to the different cell types, i.e. macrophages (11, 37) or endothelial cells (12, 38), that were used in different studies. While in vitro (11, 39) and vivo studies (40, 41) have demonstrated that CO acts as an anti-inflammatory mediator, more recent studies have questioned this paradigm by showing that bilirubin and/or Fe^{2+} chelation, both of which are increased as a consequence of HO-1 activity, play a more important role in down-regulation of adhesion molecules under inflammatory conditions (12, 13). Our own findings do not corroborate with the latter studies, as we demonstrate that CORM-3 has the propensity to inhibit VCAM-1 and E-selectin expression through the release of CO. Still, it can be argued that while CORM-3 strongly upregulates HO-1, downregulation of these adhesion molecules could occur though generation of bilirubin and ferritin-mediated Fe^{2+} chelation. Two lines of evidence are, however, against this assumption. First, pre-treatment of endothelial cells with CORM-3 did not largely influence VCAM-1 and E-selectin expression upon a subsequent challenge with TNF-α in the absence of CORM-3. Second, when induction of HO-1 was prevented by means of siRNA CORM-3 was still effective.

It must be emphasized that this study does not argue against the important role of the HO-1 system in control of inflammation, as it inherently demonstrates its anti-inflammatory potential through generation of CO, but our data do suggest that upregulation of HO-1 *per se* not necessarily exerts anti-inflammatory properties. This notion is compatible with *in vitro* studies of Foresti et al using cardiomyocytes (42). They showed that although hypoxia increased the expression of HO-1, cardiomyocytes were still vulnerable to damage during reoxygenation unless, hemin was added during the hypoxic phase. Therefore, substrate availability seems to be an important factor that influences the anti-inflammatory effect of HO-1. Production of cellular heme is a multistep process that includes mitochondrial and cytoplasmic elements. The rate-limiting step is the production of δ-aminolevulinic acid from succinyl CoA and glycine which is catalyzed by δ-aminolevulinic synthase (ALAS) (43, 44). ALAS is tightly regulated, in a negative feedback manner by heme, and positively by an increase in intracellular Ca^{2+} or proten kinase C activation (45).

Taking into account that CO has a high affinity for Fe^{2+} and acts as ligand to complete the co-ordination shell of this atom, it is likely that CO-Fe complexes will form (46, 47). Moreover, CORM-3 behaved similar as cells stably transfected with HO-1 in regard to Fe homeostasis, as demonstrated by Watts et al (48). Hence, if Fe^{2+} chelation can inhibit TNF-α mediated expression of adhesion molecules, not surprisingly, similar effects can be expected by the addition of CORM-3.

There is consistency amongst the *in vitro* studies that the major adhesion molecules that are affected by HO-1 are VCAM-1 and E-selectin (12). Nevertheless, *in vivo* studies also demonstrate down-regulation of ICAM-1, as a consequence of HO-1 expression (49, 50, 51). In addition, both basal and TNF-α induced expression of ICAM-1 are increased in *Hmox1*$^{-/-}$ compared to *Hmox1*$^{+/+}$ endothelial cells (13). Our findings are in agreement with these studies in that VCAM-1 and E-selectin were consistently downregulated by CORM-3, while ICAM-1 was marginally influenced and only in three out of eight HUVEC lines tested. In comparison, to VCAM-1 and E-selectin, ICAM-1 also behaved differently with respect to TNF-α removal. The expression of the former two adhesion molecules was completely lost 24 hrs after TNF-α removal but ICAM-1 expression was still increased at this time point.

The involvement of the NF-κB pathway as a target by which HO-1 modulates the expression of adhesion molecules seems consistent throughout the different studies (52). If the NF-κB pathway plays a pivotal role in HO-1-mediated modulation of adhesion molecules, then the question that arises is why CORM-3 not consistently affects ICAM-1 expression, although the role of NF-κB activation in the regulation of ICAM-1 has been clearly demonstrated (53, 54). Our finding that TNF-α removal results in deactivation of the NF-κB pathway indicates the importance of sustained NF-κB activation for maintaining high levels of all three adhesion molecules. Yet, deactivation of NF-κB through the addition of CORM-3 to TNF-α-stimulated endothelial cells occurred much slower and did not affect ICAM-1 expression. It thus seems that, although deactivation of NF-κB, and hence reduction in mRNA transcription, have a major effect on the expression of VCAM-1 and E-selectin, post-translational mechanisms might contribute to down-regulation of these molecules by CORM-3. In line with this, it was found that downregulation in VCAM-1 protein by TNF-α removal was much slower compared to addition of CORM-3, although NF-κB deactivation occurred much faster under the former condition.

Induction of HO-1 by CORM-3 occurred in an Nrf2-dependent manner. This is compatible with previous findings in hepatoma cells (29). Although HIF1α stabilization by CO has been described in macrophages (30), it was not involved in the induction of HO-1 in our study using endothelial cells. A number of molecules, mostly anti-oxidants, have the propensity to downregulate adhesion molecules. HO-1 might be a common denominator for the anti-inflammatory effects mediated by these anti-oxidants as most of these affect the keap1/Nrf2 pathway and result in an increased HO-1 expression (35, 55). Our study however showed that neither Nrf2 nor HO-1 was involved in downregulation of VCAM-1 expression by CORM-3.

In conclusion, our study demonstrates that CORM-3 can downregulate VCAM-1 and E-selectin even in the continued presence of TNF-α. Although HO-1 was induced by CORM-3, it did not influence the expression of adhesion molecules. We speculate that the downregulation of adhesion molecules is mediated by deactivation of the NF-κB pathway, concomitantly with an increased turn-over of these proteins. How CORM-3 influences protein turn-over is at present unclear. Our data also suggest that CORM-3 is of potential therapeutic importance in sepsis, ischemia/reperfusion injury and transplant rejection as it might resolve ongoing inflammation.

References

1. Cines, D. B., E. S. Pollak, C. A. Buck, J. Loscalzo, G. A. Zimmerman, R. P. McEver, J. S. Pober, T. M. Wick, B. A. Konkle, B. S. Schwartz, E. S. Barnathan, K. R. McCrae, B. A. Hug, A. M. Schmidt, and D. M. Stern. 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91: 3527-3561
2. Golias, C, E. Tsoutsi, A. Matziridis A, P. Makridis, A. Batistatou, and K. Charalabopoulos. 2007. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. In Vivo 21: 757-769
3. Pober, J. S., and R. S. Cotran. 1990. The role of endothelial cells in inflammation. Transplantation 50: 537-544
4. Rao, R. M., L. Yang, G. Garcia-Cardena, and F. W. Luscinskas. 2007. Endothelial–dependent mechanisms of leukocyte recruitment to the vascular wall. Circ. Res. 101: 234-247
5. Jaeschke, H., and T. Hasegawa. 2006. Role of neutrophils in acute inflammatory liver injury. Liver Int. 26: 912-919
6. Bach, F. H., W. W. Hancock, and C. Ferran. 1997. Protective genes expressed in endothelial cells: a regulatory response to injury. Immuno. Today 18: 483-486
7. Van Assche, G., S. Vermeire, and P. Rutgeerts. 2006. Focus on mechanisms of inflammation in inflammatory bowel disease sites of inhibition: current and future therapies. Gastroenterol. Clin. North Am. 35: 743-756
8. Ryter, S. W., and R. M. Tyrrell. 2000. The heme synthesis and degradation pathways: role in oxidant sensitivity: heme oxygenase has both pro- and antioxidant properties. Free Radic. Biol. Med. 28: 289-309
9. Grosser, N., S. Oberle, G. Berndt, K. Erdmann, A. Hemmerle, and H. Schroeder. 2004. Antioxidant action of L-alanine: heme oxygenase 1 and ferritin as possible mediators. Biochem. Biophys. Res. Commun. 314: 351-355
10. Erdmann, K., N. Grosser, and H. Schroeder. 2005. L-Methionine reduces oxidant stress in endothelial cells: role of heme oxygenase 1, ferritin, and nitric oxide. AAPS J 7: E195-200
11. Otterbein, L. E., F. H. Bach, J. Alam, M. P. Soares, H. Tao Lu, M. Wysk, R. J. Davis, R. A. Flavell, and A. M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat. Med. 6: 422-428
12. Soares, M. P., M. P. seldon, I. P. Gregoire, T. Vassilevskaia, P. O. Berberat, J. Yu, T. Y. Tsui, and F. H. Bach. 2004. Heme-oxygenase 1 modulates the expression of adhesion molecules associated with endothelial cell activation. J. Immunol. 172: 3553-3563
13. Seldon, M. P., G. Silva, N. Pejanovic, R. Larsen, I. P. Gregoire, J. Filipe, J. Anrather, and M. P. Soares. 2007. Heme oxygenase-1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-κB RelA phosphorylation at serine 276. J. Immunol. 179: 7840-7851
14. Maines, M. D. 1997. The heme oxygenase system: a regulator of second message gasses. Annu. Rev. Pharmacol. Toxicol. 37: 517-554
15. Maines, M. D., G. M. Trakshel, and R. K. Kutty. 1986. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J. Biol. Chem. 261: 411-419
16. Willis, D., A. R. Moore, R. Frederick, and D. A. Willoughby. 1996. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat. Med. 2: 87-90
17. Yachie, A. Y. Niida, T. Wada, N. Igarashi, H. Kaneda, T. Toma, K. Ohta, Y. Kasahara, and S. Koizumi. 1999. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase 1 deficiency. J. Clin. Invest. 103: 129-125
18. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc. Natl. Acad. Sci. USA 61: 748-755
19. Kutty, R. K., and M. D. Maines. 1981. Purification and characterization of biliverdin reductase from rat liver. J. Biol. Chem. 256: 3956-3962
20. Sethi, J. M., L. E. Otterbein, and A. M. Choi. 2002. Differential modulation by exogenous carbon monoxide of TNF alpha stimulated mitogen-activated protein kinases in rat pulmonary artery endothelial cells. Antioxid. Redox. Signal. 4: 241-248
21. Otterbein, L. E. 2002. Carbon monoxide: innovative anti-inflammatory properties of an age-old gas molecule. Antioxid. Redox. Signal. 4: 309-319
22. Neto, J., A. Nakao, K. Kimizuka, A. J. Romanosky, D. B. Stolz, T. Uchiyama, M. A. Nalesnik, L. E. Otterbein, and N. Murase. 2004. Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide. Am. J. Physiol. Renal. Physiol. 287: F979-989
23. Nakao, A., J. S. Neto, S. Kanno, D. B. Stolz, K. Kimizuka, F. Liu, F. H. Bach, T. R. Billiar, A. M. Choi, L. E. Otterbein, and N. Murase. 2005. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. Am. J. Transplant. 5: 282-291

24. Motterlini, R., J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann, and C. J. Green. 2002. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90: E17-24
25. Alcaraz, M. J., M. I. Guillen, M. L. Ferrandiz, J. Megias, and R. Motterlini. 2008. Carbon monoxide-releasing molecules: a pharmacological expedient to counteract inflammation. Curr. Pharm. Des. 14(5): 465-472
26. Foresti, R., J. Hammad, J. E. Clark, T. R. Johnson, B. E. Mann, A. Friebe, C. J. Green, and R. Motterlini. 2004. Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. British J. of Pharmacol. 142: 453-460
27. Motterlini, R. 2007. Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem. Soc. Trans. 35(Pt 5):1142-1146
28. Mannaioni, P. F., A. Vannacci, and E. Masini. 2006. Carbon monoxide: the bad and the good side of the coin, from neuronal death to anti-inflammatory activity. Inflamm. Res. 55(7): 261-273
29. Lee, B. S., J. Heo, Y. M. Kim, S. M. Shim, H. O. Pae, Y. M. Kim, and H. T. Chung. 2006. Carbon monoxide mediates heme oxygenase 1 induction via Nrf2 activation in hepatoma cells. Bochem. Biophys. Res. Commun. 343(3): 965-972
30. Chin, B. Y., G. Jiang, B. Wegiel, H. J. Wang, T. Macdonald, X. C. Zhang, D. Gallo, E. Cszimadia, F. H. Bach, P. J. Lee, and L. E. Otterbein. 2007. Hypoxia-inducible factor 1alpha stabilization by carbon monoxide results in cytoprotective preconditioning. Proc. Natl. Acad. Sci. USA 104: 5109-5114
31. Yard, B., G. Beck, P. Schnuelle, C. Braun, M. Schaub, M. Bechtler, U. Gottmann, Y. Xiao, A. Breedijk, S. Wandschneider, R. Loesel, G. Sponer, M. Wehling, and F. J. Van der Woude. 2004. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. Am. J. Transplant. 4: 22-30
32. Anrather, J., V. Csizmadia, M. P. Soares, and H. Winkler. 1999. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. J. Biol. Chem. 274: 13594-13603
33. Brouard, S., P. O. Berberat, E, Tobiasch, M. P. Seldon, F. H. Bach, and M. P. Soares. 2002. Heme Oxygenase-1 derived carbon monoxide requires the activation of transcription factor NF-κB to protect endothelial cells from tumor necrosis factor-mediated apoptosis. J. Biol. Chem. 277:17950-17961
34. Viemann, D., M. Schmidt, K. Tenbrock, S. Schmid, V. Mueller, K. Klimmek, S. Ludwig, J. Roth, and M. Goebeler. 2007. The contact allergen nickel triggers a unique inflammatory and proangiogenic gene expression pattern via activation of NF-κB and hypoxia inducible factor 1 alpha. J. Immunol. 178: 3198-3207
35. Banning, A, and R. Brigelius-Flohe. 2005. NF-κB, Nrf2, and HO-1 interplay in redox-regulated VCAM-1 expression. Antioxid. Redox Signal. 7: 889-899
36. Goebeler, M, , K. Kilian, R. Gillitzer, M. Kunz, T. Yoshimura, E. B. Broecker, U. R. Rapp, and S. Ludwig. 1999. The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. Blood 93: 857-865.
37. Chung, S. W., Y. H. Chen, and M. A. Perrella. 2005. Role of Ets-2 in the regulation of heme oxygenase-1 by endotoxin. J. Biol. Chem. 280: 4578-4584
38. Sacerdoti, D., C. Colombrita, M. H. Ghattas, E. F. Ismaeil, G. Scapagnini, M. Bolognesi, G. Li. Voltig, and N. G. Abraham. 2005. Heme oxygenase-1 transduction in endothelial cells causes downregulation of monocyte chemoattractant protein-1 and of genes involved in inflammation and growth. Cell Mol. Biol. 51: 363-370
39. Urquhart, P., G. Rosignoli, D. Cooper, R. Motterlini, and M. Perretti. 2007. Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. J. Pharmacol. Exp. Ther. 321: 656-662
40. Nakao, A., H. Toyokawa, A. Tsung, M. A. Nalesnik, D. B. Stolz, J. Kohmoto, A. Ikeda, K. Tomiyama, T. Harada, T. Takahashi, R. Yang, M. P. Fink, K. Morita, A. M. Choi, and N. Murase. 2006. Ex vivo application of carbon monoxide in University of Wisconsin solution to prevent intestinal cold ischemia/reperfusion injury. Am. J. Transplant. 6: 2243-55,
41. Akamatsu, Y., M. Haga, S. Tyagi, K. Yamashita, A. Vicente Graça-Souza, R. Ollinger, E. Czismadia, G. Aaron May, E. Ifedigbo,. L. E. Otterbein, F. H. Bach, and M. P. Soares. 2004. Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. FASEB J. 18: 771-782
42. Foresti, R., H. Goatly, C. J. Green, and R. Motterlini.2001. Role of heme oxygenase-1 in hypoxia-reoxygenation: requirement of substrate heme to promote cardioprotection. Am. J. Physiol. Heart Circ. Physiol. 281:H1976-1984
43. May, B. K., C. R. Bhasker, M. J. Bawden, and T. C. Cox. 1990. Molecular regulation of 5-aminolevulinate synthase. Diseases related to heme biosynthesis. Mol. Biol. Med. 7: 405-421
44. Kikuchi, G., and N. Hayashi. 1981. Regulation by heme of synthesis and intracellular translocation of delta-aminolevulinate synthase in the liver. Mol. Cell Biochem. 37: 27-41

45. Leffler, C. W., L. Balabanoval, A. L. fedinec, and H. Parfenova. 2005. Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. Am. J. Physiol. Heart Circ. Physiol. 289: H1442-1447
46. Watts, R. N., P. Ponka, and D. R. Richardson. 2003. Effects of nitrogen monoxide and carbon monoxide on molecular and cellular iron metabolism: mirror-image effector molecules that target iron. Biochem. J. 369(Pt 3):429-440
47. Piantadosi, C. A. 2002. Biological chemistry of carbon monoxide. Antioxid. Redox. Signal. 4: 259-270
48. Watts, R. N., and D. R. Richardson. 2004. Differential effects on cellular iron metabolism of the physiologically relevant diatomic effector molecules, NO and CO, that bind iron. Biochim. Biophys. Acta. May 28;1692(1): 1-15
49. Neto, J. S., A. Nakao. H. Joyokawa, M. A. Nalesnik, A. J. Romanosky, K. Kimizuka, T. Kaizu, N. Hashimoto, O. Azhpa, D. B. Stolz, A. M. Choi, and N. Murase. 2006. Low-dose carbon monoxide inhalation prevents development of chronic allograft nephropathy. Am. J. Physiol. Renal Physiol. 290(2): F324-334
50. Scott, J. R., D. K. Gray, A. Bihari, A. Badhwar, X. Zhang, P. Shan, P. J. Lee, S. Chakrabarti, K. A. Harris, and R. F. Potter. 2005. Heme oxygenase modulates small intestine leukocyte adhesion following hindlimb ischemia/reperfusion by regulating the expression of intercellular adhesion molecule-1. Crit. Care Med. 33(11): 2563-2570
51. Belcher, J. D., H. Mahaseth, T. E. Welch, L. E. Otterbein, R. P. Hebbel, and G. M. Vercellotti. 2006. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J. Clin. Invest. 116(3): 808-816
52. Sun, B. W., H. Sun, C. Liu, J. Shen, Z. Y. Chen, and X. Chen. 2007. Role of CO-releasing molecules liberated CO in attenuating leukocytes sequestration and inflammatory responses in the lung of thermally injured mice. J. Surg. Res. 139(1):128-35,
53. Zhou, Z., M. C. Connell, and D. J. MacEwan. 2007. TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. Cell Signal 19(6): 1238-1248
54. Collins, T., M. A. Read, A. S. Neish, M. Z. Whitley, D. Thanos, and T. Maniatis. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J. 9(10): 899-909
55. González-Gallego, J., S. Sanchez-Campos, M. J. Tunon. 2007. Anti-inflammatory properties of dietary flavonoids. Nutr. Hosp. 22(3): 287-93

Chapter 8

CORM-3 protects endothelial cells during cold preservation, resulting in improved vascular function and inhibition of intimal hyperplasia after aorta transplantation in rats

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Abstract

Carbon monoxide releasing molecules (CORMs) are a novel class of compounds that can deliver CO to tissues in a controllable fashion. Here, we investigated the beneficial effect of CORM-3 on cold preservation-induced injury *in vitro* and its influence on vascular function and on neo-intima formation after aorta transplantation.

Abdominal aortas from Lewis rats were cold preserved in UW with or without CORM-3. Hereafter, whole mount staining for CD31, acetylcholine mediated vasorelaxation and transplantation in syngenic rats were performed.

During cold preservation CORM-3 protected human umbilical vein endothelial cells (HUVEC) against necrosis and prevented denudation and intercellular gap formation in isolated rat abdominal aortas. Cold-preservation resulted in a profound loss of acetylcholine-mediated vasorelaxation of aorta segments, which was restored when CORM-3 was added during preservation. Vasorelaxation in response to the NO-donor SNP was not changed by cold preservation and not influenced by CORM-3. Cold preservation of aortic isografts in UW for 24 hrs resulted in a significant increase in fibrotic adventitial remodeling and neo-intima formation two months after transplantation compared with isografts that were not subjected to cold preservation. Presence of CORM-3 during cold preservation significantly reduced fibrotic adventitial remodeling and neo-intima formation.

Our study demonstrates that addition of CORM-3 to the UW preservation solution prevents endothelial damage, thereby limiting vascular loss of function directly after cold preservation. Because CORM-3 also prevents intimal hyperplasia, our findings might offer better approaches for organ preservation in order to limit transplant loss resulting from chronic allograft vasculopathy.

Introduction

Allocation of organ allografts has become possible by adequate improvements in organ preservation. In solid organ transplantation, static cold storage is the most widely used modality for preserving cadaveric organs prior to transplantation. However, with increasing cold ischemia time, organ quality and function are deteriorating as a consequence of a series of events such as oxidative stress, intracellular iron release and calcium overload (1-3). Cold preservation injury is a major cause of pre-tranplantation injury of allografts and is significantly associated with initial organ non-function and late transplant loss (4-7).

Chronic transplant vasculopathy remains a leading cause for chronic organ loss after transplantation (8, 9) and is characterized by a prominent vascular remodeling in which the luminal areas of arteries become obliterated due to occlusive intimal hyperplasia. Although the etiology of intimal hyperplasia is not well delineated, pre-transplantation injury is recognised as an important cause for this complication. In addition, as donor allogeneic endothelial cells of the graft's vasculature are under continuous attack of the recipient's immune system, an imbalance between damage and repair might contribute to intimal hyperplasie (10). Participation of migrating smooth muscle cells and proliferation of recruited progenitor cells in neo-intima formation have been well documented in primarily experimental models of transplant vasculopathy (11, 12).

Carbon monoxide (CO), an endogenous by-product of heme catalysis by heme oxygenase (HO), has a diversity of cell biological functions, including cGMP-mediated vasorelaxation (13, 14), inhibition of cell proliferation (15, 16), inhibition of apoptosis (17) and suppression of inflammation (18). Based on these effects it is not surprising that CO has the propensity to protect different organs against ischemia and reperfusion injury (19, 20-23). It has been postulated that the mechanism by which CO mediates organ protection is, amongst others, via the induction of HO-1 and activation of the p38 MAPK signalling pathway (13, 17, 18). In most organ transplantation studies involving CO, delivery of CO was performed by ventilation (19, 21, 22). As a novel approach to deliver CO, Motterlini et al has reported on a new class of compounds, termed CO-releasing molecules (CORMs), which are able to release CO in a controllable manner under physiological conditions (24). In particular, the fully water-soluble CORM-3 (tricarbonylchloro(glyconato)ruthenium(II)) and CORM-A1 (sodium boranocarbonate), can be potentially used as additives in preservation solutions (23, 25).

Because endothelial cell injury is a prominent feature of cold ischemia (26), strategies to mitigate cold preservation injury might be a beneficial approach to limit intimal hyperplasia. In the present study we employed *in vitro* and *in vivo* models to test the hypothesis that addition of CORM-3 to the preservation solution protects vascular endothelial cells from damage, and hence better preserves vascular function during cold storage. In addition, we hypothesize that a reduction in endothelial cell damage will significantly decrease neo-intima formation. To study the effect of CORM-3 on neo-intima formation developing as a result of solely cold preservation-induced endothelial damage and not alloantigen-driven immunity, a syngeneic Lewis-to-Lewis aortic transplant model was used.

Materials and methods

Reagents

Endothelial cell growth medium MedKIT, Phenol-red free medium (Promocell, Heidelberg, Germany), PBS (Invitrogen, Karlsruhe, Germany), Fetal bovine serum (FBS) Gold (PAA laboratories GmbH, Pasching, Austria), Tripsin/EDTA solution, DMSO, TritonX-100, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$), glycine, sodium ethoxide, tetrahydrofuran, acetylcholine (Ach), sodium nitroprusside (SNP), 1H-[1, 2, 4]oxadiazolo[4, 3-alpha]quinoxalin-1-one (ODQ) (Sigma, St. Louis, MO), Bovine Serum Albumin (SERVA, Heidelberg, Germany), 37% formaldehyde (Mallinckrodt Baker, Deventer, Holland).

Synthesis of tricarbonylchloro(glycinato)ruthenium(II)

Tricarbonylchloro(glycinato)ruthenium(II) ($[Ru(CO)_3Cl(glycinate)]$) was synthesized from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$). Briefly, $[Ru(CO)_3Cl_2]_2$ (0.5 g) and glycine (0.151 g) were placed under nitrogen in a round-bottomed flask. Methanol (291 ml) and sodium ethoxide (0.132 g) were added and the reaction was allowed to continue under stirring for 18 hrs at room temperature. The solvent was then removed under pressure and the yellow residue re-dissolved in tetrahydrofuran (THF). The yellow solution was evaporated down to give a pale yellow solid (yield 92-96%) and was aliquoted and stored in closed vials at room temperature. For each experiment CORM-3 was dissolved freshly in PBS.

Cell culture and CORM-3 treatment

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords and cultured as described previously (2). The cells were seeded in 24-well plates in basal endothelial medium supplemented with 10% FBS and essential growth factors until they formed a confluent monolayer. Cells were then stored for 24 hrs at 4^{o}C in UW solution in the presence or absence of different concentrations CORM-3. In some experiments cells were pre-treated with different concentrations CORM-3 for 6 hrs prior to cold preservation. Thereafter the cells were washed two times with PBS and stored for 24 hrs at 4°C in UW solution. Directly after cold preservation cell damage was assessed by measuring lactate dehydrogenase (LDH) (Roche Diagnostics, Mannheim, Germany) activity in the supernatants as recommended by the manufacturer. Only HUVECs from passage 2 to 6 were used in this study.

Animals

Inbred male Lewis rats weighing 250 to 300g were obtained from Charles River (Sulzfeld, Germany). Animals were kept under standard conditions and fed standard rodent chow and water ad libitum. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and were approved by the local authorities (RP Karlsruhe, AZ 35–9185.81/91/07).

Vascular function

Abdominal aortas were explanted from Lewis rat, and either directly used for subsequent experiments or stored for 24hrs at 4^{o}C in UW solution in the presence or absence of 50µM CORM-3. Vascular function measurement was performed according to previous description by Witte et al (27). Briefly, the aortas were prepared and cleaned from peri-adventitial fat and surrounding connective tissues, cut transversely into 2-mm width rings and mounted on stainless steel hooks in individual organ baths containing Krebs-Henseleit solution (NaCl 119mM, KCl 3mM, $MgCl_2$ 1.2mM, $CaCl_2$ 1.5mM, NaH_2PO_4 1.2mM, $NaHCO_3$ 20mM, Glucose 10mM) at 37 ºC and aerated with 75% N_2, 20% O_2 and 5% CO_2. The aortic rings were placed under a resting tension of 15mN and equilibrated for 60 minutes. During this period, tension was periodically adjusted to the desired level and the Krebs-Henseleit solution was changed regularly. The aortic rings were challenged by increasing concentration of KCl (20mM, 30mM, 40mM, 50mM, 80mM and 124mM) in Krebs solution in which NaCl was substituted by KCl to keep osmolarity constant. The maximal contraction forces to potassium chloride were determined according to the responsive curves. Aortic rings were washed until

resting tension was obtained again. KCl 50mM was used to pre-contract the aortic rings until a stable plateau was reached. Concentration response curves were constructed by cumulatively adding acetylcholine (Ach, 10^{-9}-10^{-4} M) or sodium nitroprusside (SNP, 10^{-9}-10^{-5} M) to elicit the endothelium-dependent (Ach) or endothelium-independent (SNP) relaxations. The data were monitored and analysed using the LabView-based software previously developed in our lab.

Whole mount aorta staining

To study the effect of cold preservation with or without CORM-3 on endothelial integrity, freshly isolated or cold preserved aortas were transversely cut into 3-4 mm segments and fixed at room temperature for 2hrs in freshly prepared 4% formaldehyde. After fixation, the segments were washed three times with PBS for 1hr. Subsequently, the segments were incubated for 20 min in PBS containing 1% BSA, 0.5% Triton and 5% rabbit serum, followed by extensively washing with PBS. The segments were incubated overnight at 4^{o}C with a purified mouse anti-rat CD31 antibody (BD Pharmingen, Heidelberg, Germany) diluted in PBS. After washing, rabbit anti-mouse Texas-red conjugated IgG was added for 2hrs. Nuclei were visualized by addition of TOTO3 10 min before mounting. Finally, the aorta segments were longitudinally cut into halves and mounted onto glass slides in 50% glycerol/PBS, with the luminal side up. Fluorescence was analysed by confocal microscopy using the corresponding excitation and emission wavelengths for Texas red (596/620nm) and TOTO3 (642/660nm).

Aorta transplantation

Aorta transplantation was performed in the syngeneic Lewis-to-Lewis strain combination. All animals received ketamine and xylazine (100 and 6 mg/kg i.p., respectively) as an anesthetic agent. A 1.5 cm long segment of the descending abdominal aorta was removed, thoroughly flushed with UW-solution, and either directly transplanted or subjected to 24 hrs of cold preservation in UW solution. Aortas were preserved in UW solution at 4°C either in the presence or absence of 50μM of CORM-3. Aortas were orthotopically transplanted in between the renal arteries and bifurcation. The cranial anastomosis was made as close to the renal arteries as technically possible to minimize diameter difference. End-to-end anastomoses were performed using 9-0 nylon suture. Total warm ischemic time in the recipient was consistently ~30 minutes. Grafts were harvested 2 months after transplantation and then processed for histological analysis as described below.

Quantification of intimal hyperplasia

Two months after transplantation, aorta grafts from each groups (n=5) were harvested, fixed in 4% formaldehyde and mounted in paraffin. Sections were cut (5 μm) from at least 4 different segments of the graft, deparaffinized according to standard methods and stained with hematoxylin/eosin.

The degree of intimal hyperplasia was assessed by morphometric analysis and expressed as mean ratio of the intima surface (μm^2) / intima + media surface (μm^2) in different sections and segments of the transplanted aorta.

Statistical analysis

Data are presented as mean ± SD or mean ± SEM for the indicated number of separate experiments. All analyses were based on more than three independent experiments. Differences between groups were determined by Student's *t* test. A *p*-value of less than 0.05 was considered statistically significant.

Results

CORM-3 protects endothelial cells against cold preservation damage

Addition of CORM-3 to the preservation solution protected endothelial cells against cold preservation damage in all HUVEC cell lines tested (n=5). The protective effect of CORM-3 was detected over a wide concentration range of CORM-3, with maximal inhibition already occurring at 16 μM (Figure 1A, black circles). To test if the protective effect of CORM-3 was dependent on the release of CO, similar concentrations of degassed CORM-3 were tested in parallel experiments. In contrast to CORM-3, degassed CORM-3 did not inhibit LDH release during cold preservation (CORM-3 vs. degassed CORM3, $p<0.01$) (Figure 1A, filled squares). We also investigated if CORM-3 pre-treatment of HUVEC, followed by cold preservation in the absence of CORM-3, was equally effective as addition of CORM-3 only during cold preservation. Pre-treatment was less effective compared to treatment during cold preservation, as significant higher concentrations of CORM-3 were required for protection (Figure 1A, open triangles). The protective effect of CORM-3 was largely independent of cGMP, since inhibition of guanylate cyclase (GC) by ODQ only marginally, though significantly, inhibited the protective effect of CORM-3 on cold preservation-induced endothelial damage (Figure 1B). Similar results were observed with higher concentrations of ODQ (data not shown).

Figure 1

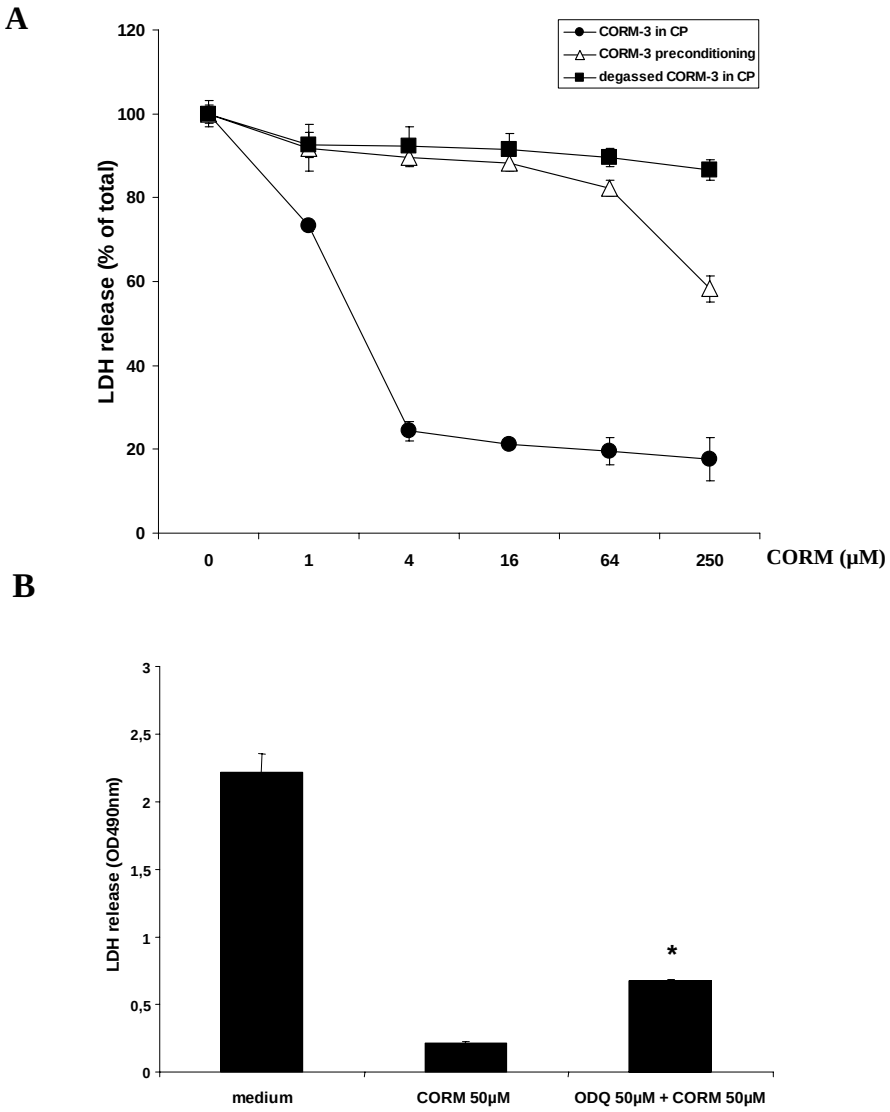


Figure 1 *CORM-3 treatment renders vascular endothelial cells resistance to hypothermia-induced cell damage.* ***(A)*** *Different concentrations of CORM-3 were either added to HUVECs during cold preservation (filled circles) or HUVECs were pre-treated for 6 hrs with similar concentrations of CORM-3 before cold preservation was started (open triangles). A degassed solution of CORM-3 (filled squares) tested in the same concentration range was used to demonstrate that the effect was mediated by CO release.* ***(B)*** *HUVECs were pre-treated for 1 hr with 50µM of ODQ or left untreated. Hereafter the cells were subjected to 24 hrs of cold preservation in the presence or absence of 50µM of CORM-3. ODQ was also present during cold preservation in the pre-treated group. Directly after cold storage cell damage was assessed by LDH-release in the supernatants. All experimental conditions were performed in triplicate. The results of a representative experiment are expressed as mean ± SD. A total of 5 experiments were performed. (* $p<0.01$)*

CORM-3 maintains vascular function after cold preservation ex vivo

We next assessed if also the endothelium in aorta segments were damaged during cold preservation and to what extent this was prevented by the addition of CORM-3 to the preservation solution. The endothelium in aorta segments that were stored for 24 hrs at 4^{o}C was dramatically changed compared with freshly isolated, no cold preserved aorta's. After cold preservation, endothelial cells displayed an irregular morphology with large intercellular gaps between the cells and in the majority of cells nuclear condensation and fragmentation occurred (Figure 2, left panel). In contrast, when CORM-3 was added during cold preservation cell morphology, CD31 expression and nuclear appearance (Figure 2, middle panel) was similar to that observed in freshly isolated aorta's that were not subjected to cold preservation (Figure 2, right panel).

Figure 2

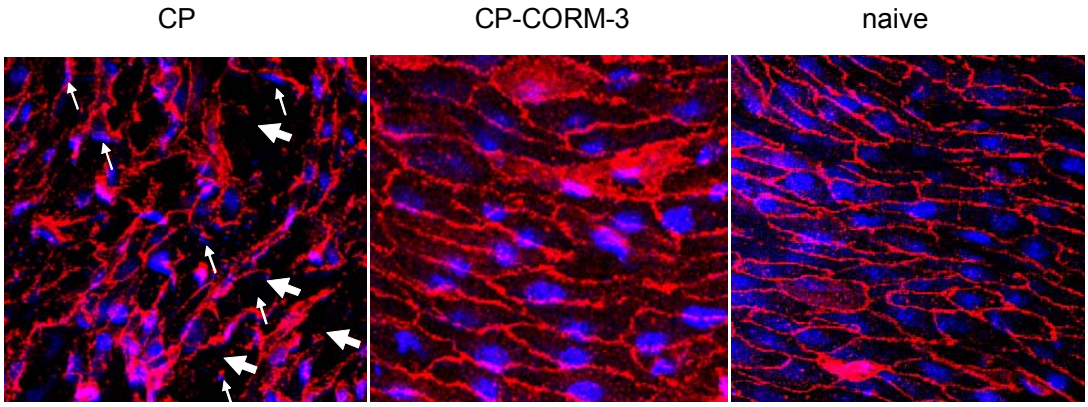


Figure 2 *Influence of CORM-3 on endothelial integrity in aorta segments subjected to cold preservation. Whole mount staining for CD31 (red) was performed as described in the Material and Method section. Nuclear staining is depicted in blue. Note that in the aorta segments subjected to cold preservation in the absence of CORM-3, endothelial cell morphology was irregular with intercellular gaps (bold arrows) appearing between adjacent endothelial cells. Also in the majority cells nuclear condensation and fragmentation (arrow) was observed (left panel). In contrast, aorta segments to which 50 µM of CORM-3 was added during cold preservation (middle panel) appeared to be similar to freshly isolated aorta segments not subjected to cold preservation (right panel). Original magnification: 200x*

Since endothelial NO production plays a critical role in vasorelaxation (27, 28), we subsequently tested if this response was impaired by cold preservation. Endothelial

dysfunction was defined as a reduction in maximal relaxation in response to acetylcholine. After 24hrs of cold preservation, vascular function was markedly impaired, as indicated by a significant reduction in maximal relaxation compared to freshly isolated aortas (aortas with cold preservation vs freshly isolated aortas: 24.6± 10.7% vs 54.1± 2.6%, $p<0.01$). When CORM-3 (50μM) was added during cold preservation, endothelial function was significantly improved, reaching a maximal relaxation of 43.4±7.6% (cold preservation-CORM-3 vs cold preservation, $p<0.05$) (Figure 3A). In contrast to the endothelium-dependent vasorelaxation response, the endothelium-independent response was not affected by cold preservation. Addition of the NO donor sodium nitroprusside (SNP) to the organ bath resulted in a dose-dependent vasorelaxation. No significant differences between the two treatment groups and freshly isolated aorta's were observed (Figure 3B), indicating that, unlike endothelial cell function, medial smooth muscle cell function is not impaired due to 24 hrs cold preservation.

Figure 3

A **B**

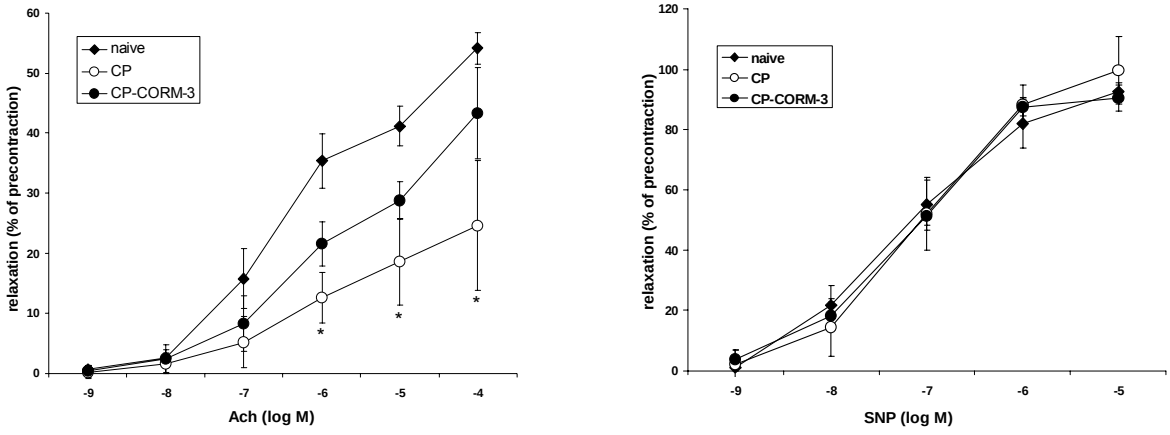
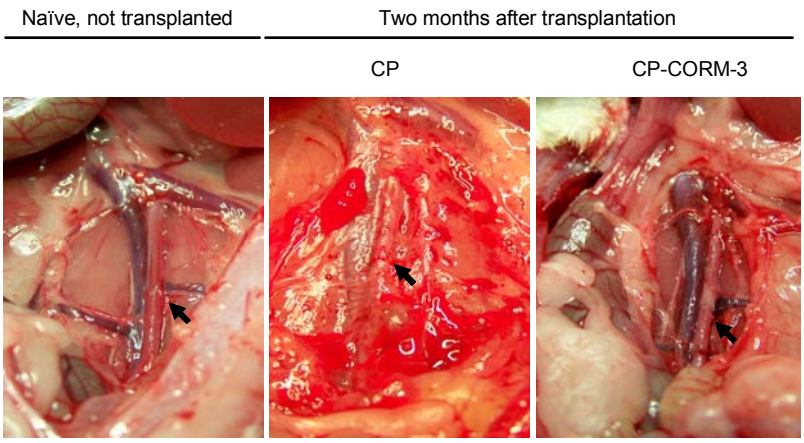


Figure 3 *Administration of CORM-3 during cold preservation improves vascular function. Isolated abdominal rat aortas were subjected to cold preservation (CP) for 24 hrs in the presence (CP-CORM-3, black circles) or absence (CP, open circles) of 50μM of CORM-3. Naïve aortas without cold preservation (black diamonds) were included as control.* ***(A)*** *Endothelial function was analysed by endothelium-dependent relaxation in response to cumulative acetylcholine concentrations (10^{-9}-10^{-4} M).* ***(B)*** *Smooth muscle cell function was demonstrated by endothelium-independent relaxation in response to cumulative sodium nitroprusside concentrations (10^{-9}-10^{-5} M). Five animals were included in each group and 16-18 aortic segments of each group were analysed. The results were expressed as mean maximal relaxation ± SEM at indicated concentration Ach or SNP. (*p<0.05, CP-CORM-3 vs CP)*

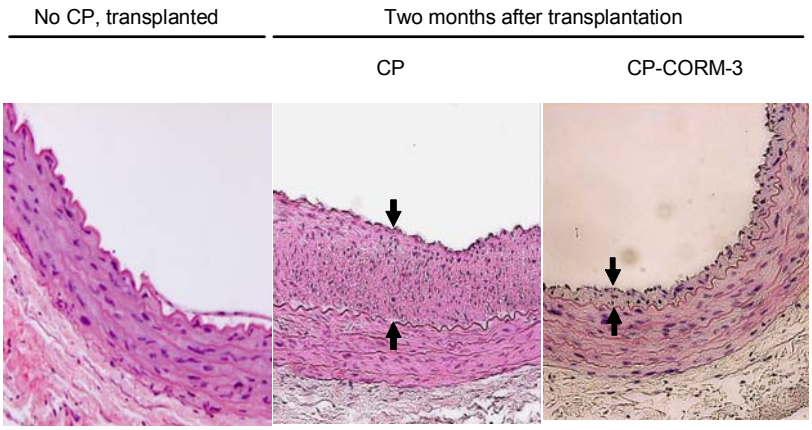
CORM-3 suppresses intimal hyperplasia after transplantation

To demonstrate the influence of cold preservation on vascular remodelling, we performed abdominal aorta transplantations in the syngeneic Lewis-to-Lewis rat strain combination. Before transplantation, donor aorta grafts were stored for 24 hrs at 4˚C in UW solution with or without 50µM CORM-3. When the aortas were harvested two months after transplantation, macroscopic inspection consistently revealed massive amounts of fibrous tissue surrounding transplants that were preserved at 4°C in the absence of CORM-3 (Figure 4A, middle panel). In contrast, grafts that were preserved in the presence of CORM-3 did not display any signs of adventitial fibrous remodeling, (Figure 4A, right panel), resembling the naïve aorta of non-transplanted animals (Figure 4A, left panel).

Figure 4 A



B



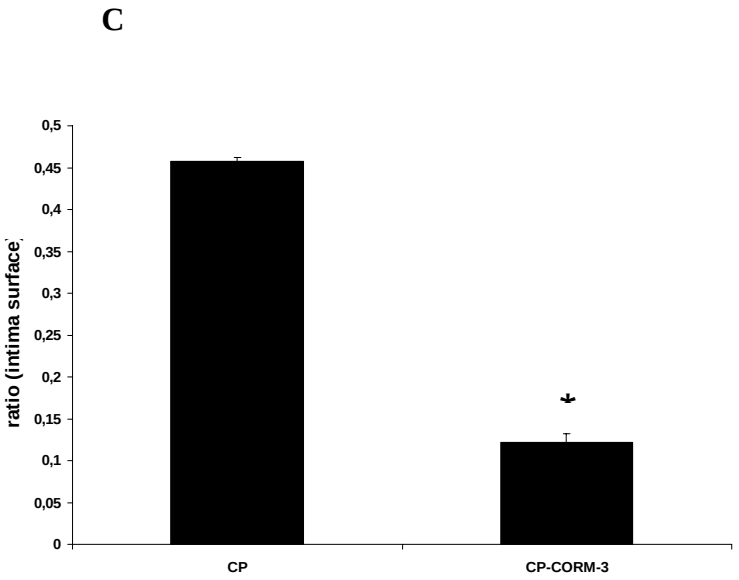


Figure 4 *Addition of CORM-3 during cold preservation inhibits vascular remodeling.* ***(A)*** *Syngeneic aorta transplantations were performed as described in the Material and Method section. The aortas were subjected to 24 hrs of cold preservation in the absence (CP) or presence (CP-CORM-3) of 50μM CORM-3. Two months after transplantation the grafts were harvested and macroscopically examined. Note the presence of fibrous tissue surrounding the graft (arrow) in the aortas subjected to cold preservation in the absence of CORM-3 (middle panel). This was not observed when 50 μM of CORM-3 was added during cold preservation (right panel). For comparison an aorta from a naïve non-transplanted rat is also depicted (left panel).* ***(B)*** *After macroscopic examination, the grafts were harvested and tissue sections were analysed by light microscopy using H&E staining. Representative photographs of transplanted grafts not subjected to cold preservation (No CP), subjected to cold preservation in the absence (CP) or presence of 50 μM of CORM-3 (CP-CORM-3) are depicted. Intimal hyperplasia is shown as the tissue in between the black arrows. Original magnification: 200x.* ***(C)*** *Quantitative analysis of intimal hyperplasia. Grafts from group CP and CP-CORM-3 were analysed by means of quantitative computerized morphometry. Grafts from the No-CP group were not analysed because intima thickening was only marginally present and only at the site of anastomosis. The degree of hyperplasia was assessed as described in the Materials and Method section. The results are expressed as mean ratio [intima surface (μm^2) / intima + media surface (μm^2)] ± SEM of 5 different sections for each group and with n=6 animals in each group. (*p<0.01).*

Histological analysis revealed that two months after transplantation neo-intima formation was marginal in the Lewis-to-Lewis model when no cold preservation was performed (Fig. 4B, picture to the left). Cold preservation significantly aggravated neo-intima formation when the aortas were preserved at 4°C in the absence of CORM-3 (Figure 4B, middle panel), while addition of CORM-3 to the preservation solution obviously attenuated neo-intima formation (Figure 4B, right panel).

Quantitative analyses using computerized morphometry (i.e. determination of the ratio of intima surface / media + intimal surface) in different sections and segments of the graft revealed that the degree of intimal hyperplasia was significantly reduced in CORM-3- treated aorta grafts (cold preservation-CORM-3 vs cold preservation, 0.12±0.01 vs 0.45±0.04, $p<0.01$) (Figure 4C).

Discussion

Chronic allograft vasculopathy (CAV) is a leading cause for late allograft loss after organ transplantation (5, 6, 10, 12). Both prolonged cold ischemia time and immune-mediated mechanisms are believed to contribute to CAV. A characteristic finding in CAV is intimal hyperplasia, arising from the migration and proliferation of smooth muscle cells (SMC) culminating in the formation of an occlusive neo-intima. In the present study we hypothesized that prevention of endothelial damage during cold preservation can significantly ameliorate intimal hyperplasia. We therefore first analysed if addition of CORM-3 to UW preservation solution can protect *in vitro* cultured HUVECs and aorta endothelium from cold preservation injury. Secondly, we assessed to what extent cold preservation affects endothelial function, defined as vasorelaxation in response to acetylcholine. Finally we addressed if prevention of vascular injury by addition of CORM-3 during cold preservation reduces intimal hyperplasia after transplantation. To exclude alloimmune-mediated mechanisms in the development of intimal hyperplasia, these latter *in vivo* studies were performed in the syngeneic Lewis-to-Lewis aorta transplant model. The main findings of our study are the following: CORM-3 protects *in vitro* cultured HUVECs as well as aorta endothelium against cold preservation-induced damage. Cold preservation causes impaired endothelial function reflected by a diminished endothelium-dependent but not -independent vasorelaxation response. Addition of CORM-3 during cold preservation improves vascular function and limits intimal hyperplasia after transplantation.

The finding that CO beneficially affects intimal hyperplasia in an aorta transplantation model is in itself not novel as this has already been demonstrated by Otterbein et al (29). However, in their model the recipients were treated, as opposed to our study in which only the graft was treated during cold preservation. Moreover recipient treatment was performed by means of CO inhalation over the whole observation period, starting directly after transplantation. In addition they did not perform cold preservation of the allograft. This is not trivial because our

data indicate that cold preservation severely damages endothelium *in vitro* as well as in aorta grafts before transplantation. Based on their data it is therefore unclear if CO treatment of the recipient is able to prevent intimal hyperplasia in the face of pre-existing vascular injury. Our study clearly shows that prevention of endothelial injury during cold preservation is very efficacious in limiting intimal hyperplasia in a syngeneic aortic transplant model. We are however aware that a syngeneic donor – recipient combination is not reflecting the clinical practice of organ transplantation and that ongoing alloimmune-mediated vascular damage is of importance for the development of intimal hyperplasia. Nevertheless, this study emphasizes the pivotal role of cold preservation damage on vascular remodeling, and suggests that if CO treatment would be implemented in transplantation medicine, it should be preferably already start during cold preservation.

Indeed, several studies have shown that application of donor CO treatment can ameliorate chronic allograft nephropathy (30) and improve islet allograft survival (31). In all of these studies, however, cold preservation was not performed. Although our data indicate that pre-treatment with CORM-3 followed by cold preservation in the absence of CORM-3 was to some extent protective, much higher concentrations of CORM-3 were required to achieve protection. Hence, the beneficial effect of donor CO conditioning might be partly lost upon prolonged cold preservation. Addition of CORM-3 to the preservation solution has also been studied in heart grafts, but they were not transplanted (25). After *ex vivo* reperfusion of these grafts, a significant improvement in systolic and diastolic function as well as coronary flow was found. Our own data with aorta grafts also show functional improvement after cold preservation when CORM-3 was added to the preservation solution. The role of cGMP in functional improvement by CORM-3 was not assessed in our study, although *in vitro* experiments revealed that the protective effect of CORM-3 on LDH-release by HUVECs was only marginally influenced in the presence of a guanylate cyclase inhibitor.

Vascular remodeling following cold preservation and transplantation was not only restricted to intimal hyperplasia, but also the amount of fibrous tissue surrounding the graft was clearly increased. Intimal hyperplasia and expansion of the fibrous tissue were both attenuated when cold preservation was performed in the presence of CORM-3. This might be related to the inhibitory effect of CO on the proliferation of smooth muscle cell (32) and fibroblasts (33). However, as ongoing inflammation might amplify smooth muscle cell proliferation and since CO has anti-inflammatory properties, inhibition of inflammation can not be completely

excluded. Specific immunohistochemisty for various leukocyte subsets was not performed since microscopical analysis of H&E stained sections did not reveal a significant amount of inflammatory cells in both the CORM-3 treated and non-treated grafts.

In conclusion, our study demonstrates that endothelial damage during cold preservation is an eligible condition for intimal hyperplasia after transplantation and that it can occur independently of an anti-donor immune response. Since chronic allograft vasculopathy remains a major cause for transplant loss, prevention of vascular injury before organ implantation is of utmost importance. This objective can simply be achieved by addition of CORM-3 to the preservation solution. Further studies are nevertheless warranted to assess the efficacy of this approach for transplantation outcome in vascularized allogeneic solid organs.

References

1. De Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med* 2003; 167 (4): 490
2. Yard, B., Beck G, Schnuelle P et al. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. Am J Transpl 2004; 4: 22
3. Ikeyama K, Sakai H, Omasa M et al. Effects of cold preservation in the lung mechanical properties in rats. Eur Surg Res 2005; 37 (2): 85
4. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int 2004; 65:713
5. Salahudeen AK. Consequences of cold ischemic injury of kidneys in clinical transplantation. J Investig Med 2004; 52:296
6. Kieran NE, Rabb H. Immune responses in kidney preservation and reperfusion injury. J Investig Med 2004; 52:310
7. Dragun D, Hoff U, Park JK et al. Prolonged cold preservation augments vascular injury independent of renal transplant immnogenicity and function. Kidney Int 2001; 60: 1173
8. Schnuelle P, Yard BA, Braun C et al. Impact of donor dopamine on immediate graft function after kidney transplantation. Am J Tranplant 2004; 4: 419
9. Schnuelle P, Berger S, de Boer J, Persijn G, van der Woude FJ. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. Transplantation 2001; 72: 455
10. Libby P, Pober JS. Chronic rejection. Immunity 2001; 14: 387
11. Hillebrands JL, Onuta G, Rozing J. Role of progenitor cells in arteriosclerosis. Trends Cardiovasc Med 2005; 15: 1
12. Hillebrands JL, Klatter FA, van der Hurk BM, Popa ER, Nieuwenhuis P, Rozing J. Origin of neo-intima endothelium and alpha-actin positive smooth muscle cells in transplant arteriosclerosis. J Clin Invest 2001; 107: 1411
13. Sammut IA, Foresti R, Clark JE et al. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. Br J Pharmacol 1998; 125:1437
14. Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ, Winslow RM. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. Circ Res 1998; 83:568
15. Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S. Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 1997; 272:32804
16. Durante W, Schafer AI. Carbon monoxide and vascular cell function. Int J Mol Med 1998; 2:255
17. Zhang X, Shan P, Otterbein LE et al. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J Biol Chem 2003; 278:1248
18. Otterbein LE, Bach FH, Alam J et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000; 6:422
19. Nakao A, Neto JS, Kanno S et al. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. Am J Transplant 2005; 5:282
20. Matsumoto M, Makino Y, Tanaka T et al. Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. J Am Soc Nephrol 2003; 14:1825
21. Kaizu T, Nakao A, Tsung A et al. Carbon monoxide inhalation ameliorates cold-ischemia reperfusion injury after rat liver transplantation. Surgery 2005; 138:229
22. Kohmoto J, Nakao A, Kaizu T et al. Low-dose carbon monoxide inhalation prevents ischemia/reperfusion injury of transplanted rat lung grafts. Surgery 2006; 140:179
23. Nakao A, Toyokawa H, Tsung A et al. Ex vivo application of carbon monoxide in university of Wisconsin solution to prevent intestinal cold ischemia/reperfusion injury. Am J Transplant 2006; 6:2243
24. Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, Green CJ. Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. Curr Pharm Des 2003; 9:2525
25. Musameh M, Green C, Mann B, Fuller B , Motterlini R. Improved myocardial function after cold storage with preservation solution supplemented with a carbon monoxide-releasing molecule (CORM-3). J Heart Lung Transplant 2007; 26(11):1192
26. Kerkweg U, Jakob M, de Groot H, Mannherz HG, Rauen U. Cold induced apoptosis of rat liver endothelial cells: contribution of mitochondrial alterations. Transplantation 2003; 76: 501
27. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. FASEB J 1989; 3: 2007
28. Vanhoutte PM. Endothelial dysfunction and atherosclerosis. Eur Heart J 1997; 18S: E19
29. Otterbein LE, Zuckerbraun BS, Haga M et al. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 2003; 9(2):183

30. Martins PNA, Reutzel-Selke A, Jurisch A et al. Induction of Carbon Monoxide in Donor Animals Prior to Organ Procurement Reduces Graft Immunogenicity and Inhibits Chronic Allograft Dysfunction. Transplantation 2006; 82: 938
31. Wang H, Lee SS, Gao W et al. Donor treatment with carbon monoxide can yield islet allograft survival and tolerance. Diabetes 2005; 54(5):1400
32. Kim HP, Wang X, Nakao A et al. Caveolin-1 expression by means of p38beta mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide. Proc Natl Acad Sci U S A. 2005; 102(32):11319
33. Zhou Z, Song R, Fattman CL et al. Carbon monoxide suppresses bleomycin-induced lung fibrosis. Am J Pathol 2005; 166(1):27

Chapter 9

General discussion

9.1 Summary

Solid organ transplantation, as a treatment modality for patients with end-stage organ disease, has become possible by adequate improvements in surgical techniques, organ preservation and immunosuppression. Organs procured from deceased donors have already suffered from damage as a consequence of brain death. Some of these donors are hemodynamic instable, which might lead to disturbances in organ perfusion (1, 2). Optimal donor management is therefore of critical importance to the success of transplantation. Apart from the deleterious event of brain death, organ allografts are further injured when in transit to the recipient. Static cold storage is the most widely used approach for organ preservation, yet allograft quality and function deteriorate with the increasing cold preservation time (3-5). The studies presented in this thesis were aimed to understand why prolonged cold preservation leads to endothelial cell injury, how this injury subsequently influences vascular function and might provoke inflammation, and finally to find strategies to prevent endothelial damage during static cold storage. With respect to the latter, we focussed on two different compounds, i.e. dopamine and related compounds and on carbon monoxide releasing molecules (CORMs).

Donor preconditioning might be a meaningful strategy to maintain organ allograft quality (7, 8). Most of the published experimental studies on donor preconditioning were aimed at reducing ischemia/reperfusion (IR) injury, a complex interrelated sequence of events that classically involves the vascular endothelium and activated leukocytes (6-9). Interestingly, donor dopamine usage is significantly associated with a reduced risk for delayed graft function, a reduced incidence of acute rejection episodes and an improvement of long-term graft survival (10-13). Usually, dopamine is given to the donor because of hemodynamic instability, but the beneficial effect of dopamine on transplantation outcome is independent of blood pressure stabilization. Recent studies from our group have demonstrated that dopamine can protect cells and tissues against cold preservation injury, which might explain, at least partially, the salutary effect of donor dopamine usage (14, 15).

Although different organs can withstand different time periods of cold ischemia before significant damage occurs, in general tissue damage increases, and hence organ quality deteriorates, with increasing cold ischemia time (3, 4, 14). As an example, cold ischemia time longer than 8-10 hrs for heart and lung allografts is considered to be undesirable while for renal allografts cold ischemia time is usually longer (3, 4, 16). One of the clinical complications of organ allografts that have been subjected to prolonged cold ischemia time is

the loss of the endothelial barrier function (17-20). *In vitro* dopamine can not completely prevent endothelial barrier dysfunction during cold preservation. Nevertheless, this treatment restores barrier function rapidly upon rewarming (15).

In **Chapter 2,** we employed a rat model to test the hypothesis that dopamine treatment ameliorates tissue damage associated with hypothermic preservation and reperfusion of lung allografts. As tissue oedema is a consequence of barrier dysfunction, resulting in an increase in lung weight and an increase in pulmonary arterial and inspiratory pressure (21), we used these parameters as read out for barrier dysfunction and assessed the effect of different time periods of cold storage followed by reperfusion in an isolated ventilated and perfused lung model. In line with our previous *in vitro* findings (15), this study showed that a cold preservation time longer than 6 hrs resulted in profound oedema formation. Dopamine pre-treatment significantly inhibited oedema formation as was reflected by a decrease in peak inspiratory pressure (PIP), pulmonary arterial pressure (PAP) and lung weight compared to lungs obtained from untreated rats. These findings have also been reported for human lung allografts that were not suitable for transplantation (22). In addition, dopamine pre-treatment significantly abrogated CINC-1 production, the upregulation of adhesion molecules during reperfusion was likewise inhibited. Since pulmonary oedema largely contributes to poor lung function after transplantation (23), implementation of donor dopamine in donor management might be considered even in hemodynamic stable donors. Our group finished recently a prospective randomized multi-centre study on the beneficial effect of donor dopamine usage in hemodynamic stable donors on delayed graft function after renal transplantation. Analysis of the data revealed that dopamine treatment significantly reduced the risk for delayed graft function, this effect was more pronounced when cold ischemia time was long. Whether other organ allografts also have been benefited from dopamine is currently being investigated in this study.

It also remains to be addressed if the effect of dopamine in the isolated and ventilated lung model was receptor mediated or not. Several studies have indicated that the Na/K ATPase plays an important role in fluid handling in lungs. Stimulation of β-adrenergic receptors activates the Na/K ATPase (24). Therefore, unlike in the *in vitro* studies, the effect of dopamine might be receptor-mediated. Further studies are required to elucidate this issue.

In order to understand the beneficial effect of dopamine on cold preservation injury, in **Chapter 3,** we employed an *in vitro* model and investigated the changes in intracellular ATP concentration, redox balance and Ca^{2+} homeostasis during hypothermic preservation and how

this was influenced by dopamine pre-treatment. Our study demonstrated that increased oxidative stress, reflected by the occurrence of a redox imbalance, changes in Ca^{2+} homeostasis and depletion of intracellular ATP are major cellular events that are associated with hypothermic preservation. As demonstrated previously, dopamine pre-treatment renders endothelial cells transient resistance to hypothermia-induced damage. Dopamine pre-treatment furthermore prevents the occurrence of a redox imbalance and mitochondrial [Ca^{2+}] overload. Also ATP depletion is retarded during cold preservation in dopamine treated endothelial cells. Based on the results, we postulate that mitochondrial [Ca^{2+}] overload is a key event in cold preservation injury and that perhaps prevention of this by dopamine might explain the protective effect of dopamine. As the protective effect of dopamine is strictly redox-dependent, we must assume that the increase in intracellular [Ca^{2+}] during hypothermic preservation is initiated by a redox imbalance. It has been demonstrated in other studies that depletion of glutathione (GSH) activates ryanodin receptors in the endoplasmic reticulum and consequently Ca^{2+} is released from intracellular stores (25, 26). Subsequently activation of store-operated calcium channels will result in an influx of Ca^{2+} from the extracellular milieu (27-29). This sequel of evens is compatible with two important observations described in **Chapter 3**: firstly dopamine prevents depletion of SH reduction equivalents, and secondly depletion of Ca^{2+} from the intracellular stores by thapsigargin treatment can overcome the protective effect of dopamine. However, the release of Ca^{2+} from the intracellular stores is not sufficient to drive cell death, because thapsigargin treatment can not overcome the protective effect of EDTA. The reason why ATP is depleted much faster in untreated endothelial cells might be the consequence of ATP exhaustion to maintain Ca^{2+} homeostasis or might be the result of cell leakage.

This study did not address why depletion of SH reduction equivalents occurred during cold preservation. However, as deferoxamine (30, 31) or HO-1 over-expression (32) have shown to be also protective during cold preservation, we postulate that an increase in the chelatable intracellular iron pool (30) might give rise to hydroxyl radicals by Fenton chemistry. These radicals, in turn might convert GSH to GSSG. In the presence of intracellular dopamine, the hydroxyl groups of the catechol moiety of dopamine can scavenge hydroxyl radicals and thus prevent oxidation of GSH.

Inasmuch as our study indicates that prevention of a Ca^{2+} influx during cold preservation might be beneficial, it does not imply that Ca^{2+} should be completely omitted from

preservation solutions. Omission of Ca^{2+} from the extracellular milieu can lead to opening of unselective cation channels and hence to membrane depolarization (33, 34). In fact, addition of small amounts of Ca^{2+} to preservation solutions has proven to be more protective in experimental liver transplantation (33, 34).

If we appreciate that dopamine can prevent intracellular Ca^{2+} accumulation in a redox-dependent manner, the next question to be addressed is then what are the structural requirements for dopamine to be protective. Since we already have demonstrated that the protective effect of dopamine is not receptor-mediated, occurs relatively fast, is not specific for dopamine but also holds true for other dihydroxyphenolic compounds and does not require *de novo* protein synthesis (14, 15), we postulated that structural entities within these compounds mediate protection. Further studies to elucidate the structure-function relationship with respect to protection against cold preservation damage are described in **Chapter 4**. In this study we analysed the protective potency of a series of related compounds that either differ in their relative hydrophobicity or in the position of the dihydroxyl groups. We could demonstrate that the relative hydrophobicity, expressed as LogP value, tightly correlates with the efficacy of protection. The importance of the relative hydrophobicity is that it facilitates cellular uptake. However, hydrophobicity alone is not sufficient to mediate protection, because protection also requires reducing substituents on the benzene ring. This is emphasized by the fact that only ortho- or para- positioned hydroxyl groups on the bezene ring will yield protective compounds. Meta-dihyroxybenzene or benzoic acids do not convey protection even when the hydrophobicity was increased by covalent coupling of a fatty acid. Ortho- and para-dihydroxybenzenes are known to be strong reducing agents due to the ease of quinone formation, while the meta-dihydroxyl derivatives have no radical scavenging potential since oxidation of the hydroxyl groups does not occur under normal conditions.

These two structural entities found in our study together with the data presented in **Chapter 3** may promote the understanding of the mechanisms involved in preservation injury of organ allografts and might eventually lead to the use of more effective compounds for organ preservation that are devoid of hemodynamic action. Since acylated dopamine derivatives and alkyl dihydroxybenzamides have been demonstrated to be anti-inflammatory agents (35, 36), clinical application of these compounds seems to be a promising strategy not only for prevention of pre-transplantation injury in donor organs but also to ameliorate inflammation after transplantation.

During cold preservation degradation of cytosolic proteins has been reported (15, 37). Proteolysis is generally mediated by activation of a variety of enzymes. This might occur as a consequence of ATP depletion and an increase in cytosolic calcium. In particular, activation of the Ca^{2+}-dependent proteases, i.e. calpains, seems to be corollary to the imbalance in intracellular calcium homeostasis during cold storage (38, 39). The ubiquitin proteasome system (UPS) (40) and caspases (41) might be equally involved in proteolysis during cold preservation. In **Chapter 5,** we investigated to what extent cold storage changes the cellular proteome and if activation of these proteolytic pathways occurred in HUVEC during cold storage. Our findings demonstrated that indeed activation of the calpain pathway and the ubiquitin proteasome system occurs under cold preservation conditions. In dopamine pre-treated cells activation of the calpain pathway did not occur, however, ubiquintination was not influenced by dopamine. Neither proteasome inhibitors nor an inhibitor of calpain 1 were able to prevent cell death during hypothermic preservation. These data thus suggest that although proteolysis might occur during cold preservation, inhibition of single proteolytic pathways is not effective to prevent cell death during cold storage. It must be noted, that inhibition of multiple proteolytic pathways was not tested in this study.

According to danger hypothesis, tissue injury of allografts is not only recognized in the recipient by the immune system, but also evokes immune activation (42). Hypothermic preservation induces cell necrosis and tissue damage in a time-dependent fashion (14, 43). Nevertheless it is not yet clear how hypothermic preservation might cause immune activation. In **Chapter 6,** we therefore investigated if soluble factors were released upon hypothermic preservation that might signal to the innate immune system. We focussed on HMGB1 and adenosine, two important molecules known to modulate innate immunity (44-47). We showed that expression of HMGB1 is completely lost in endothelial cells subjected to cold preservation, but not when the cells were rendered resistant by dopamine pre-treatment. HMGB1 was bioactive, indicated by the findings that supernatants of damage cells were able to upregulate the expression of adhesion molecules and the production of IL-8 in an HMGB1-dependent fashion. However, the unexpected finding that the same supernatants were also able to downregulate TNF-α production in LPS stimulated whole blood assays suggested that other factors were likely present. Subsequent experiments revealed that adenosine is also released during hypothermic preservation and that adenosine was responsible for the inhibitory effect on TNF-α production. While the release of HMGB1 only occurred in damaged cells, the release of adenosine was independent of cell damage. Because adenosine

not only modulates innate immunity but also improves endothelial barrier function (48, 49), adequate donor treatment might thus prevent the release of HMGB1 but might preserve the action of adenosine on the endothelium.

Because HMGB1 and adenosine are released during cold preservation and organ allografts are flushed before implantation, it can be argued that these *in vitro* findings have no relevance for organ transplantation. Endothelial cells express the receptor for advanced glycation end-products RAGE (50), a putative receptor for HMGB1, which might already bind its ligand during cold preservation. Subsequent warm reperfusion then can cause receptor activation leading to phenotypic and functional changes in endothelial cells. The relevance of adenosine release during hypotherimia is indeed questionable as adenosine is added to some preservation solutions, e.g. UW.

Leukocyte extravasation, as occurring during inflammation, is a highly regulated process in which adhesion molecules expressed on the endothelial cell-surface interact with their ligands on leukocytes (51). In addition, leukocytes are recruited to the sites of inflammation via endothelial production of chemokines. Up-regulation of these inflammatory mediators facilitates leukocyte migration and subsquently amplifies inflammation (52). Understanding of the mechanisms that control inflammation are of utmost importance to identify putative targets for the development of anti-inflammatory drugs. In recent years, a new class of molecules, termed carbon monoxide releasing molecules (CORM), has been described that are capable of liberating CO under appropriate conditions. In particular, CORM-3 [tricarbonylchloro(glyconato)ruthenium(II)] and CORM-A1 (sodium boranocarbonate), which both are fully water-soluble, rapidly liberate CO when dissolved in physiological solutions (53). **In Chapter 7**, we studied how CORM-3 modulates the expression of adhesion molecules on endothelial cells and if HO-1 mediated-perpetuation was involved. Our results showed that CORM-3 consistently inhibited the upregulation of VCAM-1 and E-selectin on TNF-α stimulated HUVEC, partly due to the deactivation of NFκB. Interestingly, down-regulation of VCAM-1 and E-selectin expression by CORM-3 even occurred when CORM-3 was added 24 hrs after TNF-α stimulation. Sustained expression of VCAM-1 required the continuous presence of TNF-α. TNF-α removal was more effective in the reduction of VCAM-1 mRNA level, but VCAM-1 protein was down-regulated more rapidly when CORM-3 was added compared to TNF-α removal. This suggests that the modulation of VCAM-1 by CORM-3 most likely also occurred post-transcriptionally. CORM-3 itself up-regulated HO-1

in an Nrf2 dependent fashion, however, HO-1 expression did not significantly contribute to the effect by CORM-3. Neither in HO-1- nor in Nrf2-siRNA treated HUVEC the efficacy of CORM-3 to down-regulate VCAM-1 expression was lost.

As discussed already, prolonged cold ischemia is significantly associated with initial organ non-function and late transplant loss (54, 55). Chronic transplant vasculopathy remains a leading cause for chronic organ loss after transplantation (54, 56). Intimal hyperplasia and arterial obliteration are prominent features of chronic vasculopathy. In **Chapter 8**, we investigated the beneficial effect of CORM-3 on hypothermia-induced injury in HUVECs and its influence on vascular remodelling and vascular function in syngeneic rat aorta transplantation model. Our data showed that CORM-3 protected endothelial cells against hypothermia-mediated injury via liberation of CO. Cold storage induced endothelial denudation and intercellular gap formation in isolated rat abdomen aortas, while this was prevented when CORM-3 was added to the preservation solution. Similarly, vascular function was significantly impaired after 24 hrs of cold storage. This was largely due to impairment of endothelia-mediated NO production, since the relaxation response by addition of SNP was not affected after cold storage. In line with the observation that CORM-3 prevented endothelial denudation, we could demonstrate that addition of CORM-3 during cold storage also better preserved vascular function. Two months after aorta transplantation in syngeneic rats, neo-intima was significantly increased in aortas that were subjected to 24 hrs of cold preservation. When CORM-3 was added during preservation, neo-intima formation was significantly abrogated.

9.2 Conclusions and future perspectives

Based on the finding that kidney transplants from unrelated living donors are performing exceedingly well despite poor HLA compatibility (57), the concept of pre-transplantation tissue injury as an important risk factor for long-term allograft survival has been more appreciated in recent years. Since cold preservation tissue damage is a major cause of pre-transplantation injury, prevention of this type of damage is regarded as an effective approach to improve transplantation outcome (3-5). Damage of the vascular endothelium is a prominent feature of cold ischemia (58). This may facilitate inflammation through the release of HMGB1 and through impairment of the endothelial barrier function.

To meet the growing demand of organ allografts and relative stable supply of cadaveric organ donors, the use of organs from so called "marginal" donors has increased dramatically. Yet, organs obtained from these donors might be more susceptible to cold preservation injury, resulting in a higher rate of delayed graft function (59) and possibly in a decrease in allograft-survival (60). Therefore better ways to preserve organs, or as suggested from the data presented in this thesis, new strategies to prevent cold preservation injury are warranted.

Although in the past dopamine has been frequently used in hemodynamic instable donors, in more recent years dopamine has been largely abandoned at the ICU because the beneficial effect of dopamine on renal function could not be demonstrated (61). However, our recently finished prospective randomized multi-centre study, together with the data presented in this thesis strongly favors the use of dopamine in donor management. Nevertheless, the question whether we should use hemodynamic active compounds to achieve a better organ quality after prolonged preservation is genuine and deserves more attention. Since the beneficial effect of dopamine on cold preservation injury is independent of its hemodynamic action, the use of dopamine-like compounds that are devoid of blood pressure stabilizing effects, e.g. n-octanoyl-doapmine (NOD) would be more appropriate in hemodynamic stable donors. Also in non heart beating donors, the use of positive inotropic agents is not desirable. Renal allografts from these donors are more prone to develop delayed graft function after transplantation.

Inasmuch as our *in vitro* data suggest that the use of NOD as donor pre-conditioning strategy might be more favorable than dopamine, the hydrophobic character of NOD makes its application more difficult. Ways to deliver NOD to donor organs are therefore of equal importance in future research. One possible strategy to overcome this problem is the use of so-called semifluorinate alkanes (SFA) emulsions. SFA's are clinically used in eye surgery as temporal tamponade. Interestingly they have the propensity to act as solvent for a number of hydrophobic agents (62). Preliminary data from our group indicate that NOD can be dissolved in SFA without losing its protective effect on cold preservation injury *in vitro* (unpublished data). Moreover SFA emulsions have anti-inflammatory properties as they inhibit TNF-α production in whole blood assays. We could also demonstrate in a model of warm ischemia-mediated acute renal failure that intravenous application of these emulsions significantly inhibited the rise in serum creatinine and that recovery of renal function was much faster when SFA's were applied prior to ischemia (unpublished data).

Maintaining good organ quality during cold preservation can also be achieved by addition of CORM to the preservation solution. We have not studied the efficacy of this approach in vascularized organs, and thus the conclusions reach in this study must be interpreted with some pre-caution.

In conclusion, this thesis demonstrates the detrimental influence of cold preservation and unveils some of the processes that might be involved in cell damage. Moreover this thesis provides experimental evidence on how to prevent this type of injury. In the light of the increasing shortage of cadaveric donors, attempts should be made in future research to keep transplant recipients from re-entering the waiting list as long as possible. Because chronic transplant loss is partly attributable to prolonged cold ischemia time, either logistical change to reduce cold ischemia time, i.e. local donors for local patients, or donor pre-conditioning to adapt organ allografts to cold ischemia changes should be considered.

References

1. Novitzky D. Selection and management of cardiac allograft donors. Curr Opin Cardiol. 11:174-182, 1996
2. Shivalkar B, van Loon J, Wieland W, Tjandra-Mage TB, Borgers M, Plets C, Flameng W. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. Circulation 1993; 87:230-239,
3. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney Int. 2004; 65:713-8
4. Salahudeen AK. Consequences of cold ischemic injury of kidneys in clinical transplantation. J Investig Med. 2004; 52:296-8
5. Kieran NE, Rabb H. Immune responses in kidney preservation and reperfusion injury. J Investig Med. 2004; 52:310-4
6. Entman ML, Smith CW. Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. Cardiovasc Res. 1994; 28:1301-1311
7. Fabre JW. The role of polymorphic donor peptides in allograft recognition and rejection. Immunol Rev. 1996; 154:21-43
8. Lefer AM. Role of selectins in myocardial ischemia-reperfusion injury. Ann Thorac Surg. 1995; 60:773-777
9. Paller MS. The cell biology of reperfusion injury in the kidney. J Investig Med. 1995; 42:632-639
10. Schnuelle P, Berger S, de Boer J, Persijn G, van der Woude FJ. Effects of catecholamine application to brain dead donors on graft survival in solid organ transplantation. Transplantation 2001; 72:455-463
11. Schnuelle P, Lorenz D, Mueller A, Trede M, van der Woude FJ. Donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. Kidney Int 1999; 56:738-746
12. Goettmann U, Notheisen A, Brinkkoetter PT, Yard BA, Waldherr R, schnuelle P, van der Woude FJ, Braun C. Influence of donor pre-treatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 2005; 79:1344-1350
13. Schnuelle P, Yard BA, Braun C, Dominguez-Fernandez E, Schaub M, Birck R, Sturm J, Post S, van der Woude FJ. Impact of donor dopamine on immediate graft function after kidney transplantation. Am J Transplant. 2004; 4:419-426
14. Yard B, Beck G, Schnuelle P et al. Prevention of cold-preservation injury of cultured endothelial cells by catecholamines and related compounds. Am J Transplant 2004; 4:22-30
15. Brinkkoetter PT, Beck GC, Goettmann U et al. Hypothemia-induced loss of endothelial barrier function is restored after dopamine pre-treatment: role of p42/p44 activation. Transplantation 2006; 82:534-542
16. De Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med. 2003; 167 (4): 490-511.
17. Hidalgo MA, Shah KA, Fuller BJ et al. Cold ischemia-induced damage to vascular endothelium results in permeability alterations in transplanted lungs. J Thorac Cardiovasc Surg 1996;112:1027
18. Carbognani P, Spaggiari L, Rusca M et al. Pulmonary endothelial cell modifications after cold storage in solid organ preservation solutions. J Int Med Res 1995; 23: 200-206
19. Pickford MA, Manek S, Green CJ et al. The pathology of rat lung isografts following 48 or 72-hour cold storage and subsequent reperfusion in vivo for up to 1 month. Int J Exp Pathol 1992; 73: 685-697
20. Hall SM, Odom N, McGregor CG, et al. Transient ultrastructural injury and repair of pulmonary capillaries in transplantated rat lung: effect of preservation and reperfusion. Am J Respir Cell Mol Biol 1992; 7:49
21. Wang LS, Yoshikawa K, Miyoshi A et al. The effect of ischemic time and temperature on lung preservation in a simple ex vivo rabbit model used for functional assessment. J Thorac Cardiovasc Surg 1989; 98: 333-342
22. Ware LB, Fang X, Wang Y, Sakuma T, Hall TS, Matthay MA. Selected contribution: mechanisms that may stimulate the resolution of alveolar oedema in the transplantated human lung. J Appl Physiol 2002; 93(5):1869-1874.
23. McCowin M, Hall TS, Babcock W, Solinger LL, Hall KW, Jablons D. The impact of changes in radiographic abnormalities in organ donors on successful lung donation. J Heart Lung transplant 2001; 20(2):242-243
24. Scheid CR, Honeyman TW, Fay FS. Mechanism of beta-adrenergic relaxation of smooth muscle. Nature 1979; Jan 4;277(5691):32-6.
25. Pessah IN. Ryanodine receptor acts as a sensor for redox stress. Pest Manag Sci 2001; 57: 941-5
26. Koshita M, Miwa K, Oba T. Sulfhydryl oxidation induces calcium release from fragmented sarcoplasmic reticulum even in the presence of glutathione. Experientia. 1993; 49(4):282-4
27. Groschner K, Hingel S, Lintschinger B, Balzer M, Romanin C, Zhu X, Schreibmayer W. Trp proteins form store-operated cation channels in human vascular endothelial cells. FEBS Lett. 1998; 437(1-2):101-6

28. Harder DR: Ca-induced Ca release: lessons regarding cell models. Am J Physiol Heart Circ Physiol 2004; 287: H1885-1886
29. Sparagna GC, Gunter KK, Sheu SS, Gunter TE: Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. J Biol Chem 1995; 270: 27510-27515
30. Kerkweg U, Li T, de Groot H, Rauen U: Cold-induced apoptosis of rat liver cells in University of Wisconsin solution: the central role of chelatable iron. Hepatology 2002; 35: 560-567
31. Salahudeen AK: Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 2004; 287: F181-187
32. Sikorski EM, Hock T, Hill-Kapturczak N, Agarwal A. The story so far: Molecular regulation of the heme oxygenase-1 gene in renal injury. Am J Physiol Renal Physiol 2004; 286: F425-441
33. Ben Abdennebi, H. et al. A preservation solution with polyethylene glycol and calcium: a possible multiorgan liquid. Transpl Int 2002; 15: 348-354
34. Dutkowski, P. et al. Calcium prevents loss of glutathione and reduces oxidative stress upon reperfusion in the perfused liver. Int J Surg Investig 2000; 2: 1-7
35. Loomans, M. E., Janusz, J. M. & Buckwalter, B. (1986) GB 2168976
36. Takita, H., Noda, S., Mukaida, Y., Inada, K., Toji, M., Kimura, F., Nitta, T., Watanabe, K., Umekawa, K. & Kobayashi, H. (1985) EP 0142241
37. Charrueau C, Blonde-Cynober F, Coudray-lucas C et al. Prevention of proteolysis in cold-stored rat liver by addition of amino acids to the preservation solution. J Gastroenterol Hepatol 2000; 15: 1199-204
38. Kohli V, Gao W, Camargo CA, Jr., Clavien PA. Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. Proc Natl Acad Sci U S A 1997; 94 (17): 9354
39. Goll DE, Thompson VF, Li H, Wei W, Cong J. The Calpain System. Physiol Rev 2003; 83: 731
40. Majetschak M, Patel MB, Sorell LT, Liotta C, Li S, Pham SM. Cardiac proteasome dysfunction during cold ischemic storage and reperfusion in a murine heart transplantation model. Biochem Biophys Res Commun 2008; 365(4):882-8
41. Jani A, Ljubanovic D, Faubel S, Kim J, Mischak R, Edelstein CL. Caspase inhibition prevents the increase in caspase-3, -2, -8 and -9 activity and apoptosis in the cold ischemic mouse kidney. Am J Transplant 2004; 4: 1246-54
42. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994; 12:991-1045
43. Bartels-Stringer M, Kramers C, Wetzels JF, Russel FG, Groot H, Rauen U. Hypothermia causes a marked injury to rat proximal tubular cells that is aggravated by all currently used preservation solutions. Cryobiology 2003; 47:82-91
44. Mueller S, Scaffidi P, Degryse B et al, New EMBO member's review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J 2001; 20:4337-40
45. Dumitriu IE, BarauH P, Manfredi AA, Bianchi ME, Rovere-Querini P. HMGB1: guiding immunity from within. Trends Immunol 2005; 26:381-7
46. Popovic PJ, DeMarco L, Lotze MT et al. High mobility group B1 protein suppresses the human plasmacytoid dendritic cell response to TLR9 agonists. J Immunol 2006; 177:8701-7
47. Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. J Appl Physiol 1994; 76:5-13
48. Eltzschig HK, Iba JC, Furuta GT, et al. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. J Exp Med. 2003; 198:783-96
49. Eltzschig HK, Weissmuller T, Mager A, Eckle T. Nucleotide metabolism and cell-cell interactions. Methods Mol Biol. 2006; 341:73-87
50. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. Circ Res 2004; Aug 6;95(3):233-8
51. Cines, D. B., E. S. Pollak, C. A. Buck et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998; 91: 3527-3561
52. Rao, R. M., L. Yang, G. Garcia-Cardena, and F. W. Luscinskas. Endothelial–dependent mechanisms of leukocyte recruitment to the vascular wall. Circ Res 2007; 101: 234-247
53. Alcaraz, M. J., M. I. Guillen, M. L. Ferrandiz, J. Megias, and R. Motterlini. Carbon monoxide-releasing molecules: a pharmacological expedient to counteract inflammation. Curr Pharm Des 2008; 14(5): 465-472
54. Schnuelle P, Yard BA, Braun C, et al. Impact of donor dopamine on immediate graft function after kidney transplantation Am J Transplant 2004; 4: 419-26
55. Gottmann U, Notheisen A, Brinkkoetter PT, et al. Influence of donor pretreatment with dopamine on allogeneic kidney transplantation after prolonged cold storage in rats. Transplantation 2005; 79 (10): 1344-53
56. Schnuelle P, Berger S, de Boer J, Persijn G, van der Woude FJ. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. Transplantation 2001; 72: 455-63
57. Terasaki PI, Gjertson D, Cecka JM, Takemoto S.Fit and match hypothesis for kidney transplantation. Transplantation 1996; 62:441-445

58. Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 2004; 287: F181-187
59. Nyhof E, Wiehl S, Steinhoff J, Krüger S, Mueller-Steinhardt M, Fricke L. Relationship between donor factors, immunogenic up-regulation, and outcome after kidney transplantation. Transplant Proc 2005; 37:1605-7
60. Parzanese I, Maccarone D, Caniglia L, Pisani F, Mazzotta C, Rizza V, Famulari A. Risk factors that can influence kidney transplant outcome. Transplant Proc 2006 38; 1022-3
61. Day NP, Phu NH, Mai NT et al. Effects of dopamine and epinephrine infusions on renal hemodynamics in severe malaria and severe sepsis. Crit Care Med 2000; 28: 1353-62
62. El Abed AI, Ionov R, Goldmann M. Structural and electric properties of two semifluorinated alkane monolayers compressed on top of a controlled hydrophobic monolayer substrate. Phys Rev E Stat Nonlin Soft Matter Phys 2007; 76(4 Pt 1):041606

Nederlandse samenvatting

Orgaantransplantatie neemt een steeds belangrijkere positie in bij de behandeling van terminaal orgaanfalen. Verbeteringen van chirugische technieken, de introductie van nieuwe immuun supressive medicamenten alsmede de verbetering van orgaan preservatie hebben er toe geleid dat de transplantaat overleving in de loop der jaren sterk verbeterd is. Desalnietemin is het chronische verlies van transplantaten nauwelijks afgenomen.

Het merendeel van getransplanteerde organen zijn afkomstig van gestorven donoren. Deze hebben in vergelijking met organen van levende donoren het nadeel dat ze reeds voor implantatie mogelijk beschadigd zijn als gevolg van hersendood en preservatie. De meest gebruikte orgaan preservatie methode vandaag de dag is de zogenaamde „static cold storage“ methode. Afhankelijk van de koude preservatie tijd, neemt de kwaliteit van de organen af, hetgeen de functie na transplantatie negatief beinvloedt.

De studies die in dit proefschrift zijn beschreven houden zich bezig met de vraagstelling waarom een lange koude preservatie duur leidt tot schade aan het endotheel, hoe deze schade vervolgens een ontstekings reactie kan beinvloeden en hoe verschillende stoffen de schade aan het endotheel tijdens koude preservatie kunnen voorkomen. Daarbij zijn twee stoffen onderzocht, te weten dopamine en zogenaamde „carbon monoxide (CO) releasing molecules (CORM)“.

De resultaten van deze studies laten zien dat bij een lange koude preservatie duur belangrijke intracellulaire systemen worden verstoord. Zowel dopamine als ook CORM kunnen deze verstoring gedeeltelijk voorkomen en het kwaliteitsverlies van organen tegen gaan. Daarnaast heeft CORM zogenaamde anti-inflammatoire eigenschappen, die ertoe kunnen bijdragen dat pathologische veranderingen in de bloedvaten na transplantie, zoals intima hyperplasie, kunnen worden voorkomen.

Color figures

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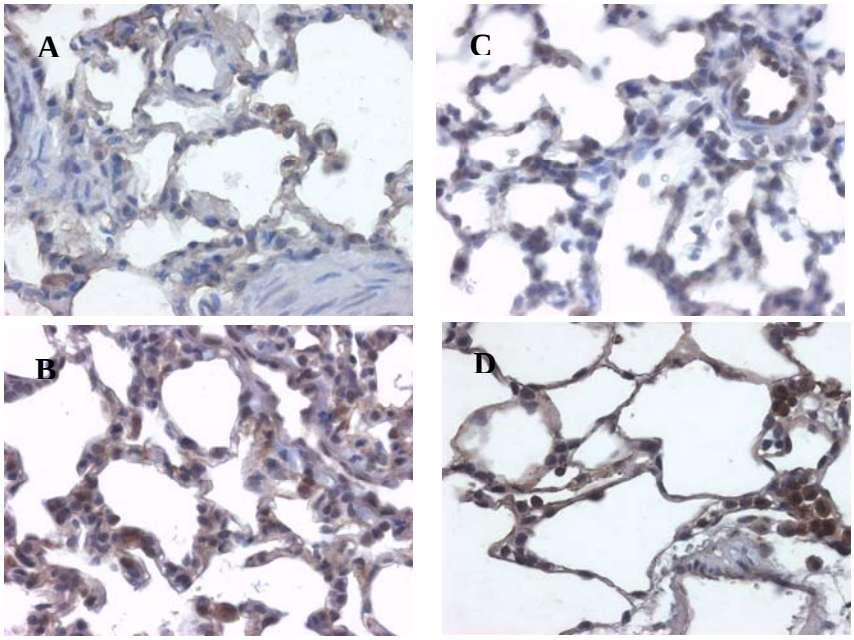

Figure 4 Influence of dopamine treatment on ICAM expression. *Rats were treated as described in Figure 1, with dopamine (A and C) or left untreated (B and D). Hereafter the lungs were directly reperfused and ventilated for 3 hr (A and B) or subjected to 8 hr of cold preservation before reperfusion and reventilation (3hr) was initiated. The results of a representative experiment (n=5) is depicted. Original magnification was X40.*

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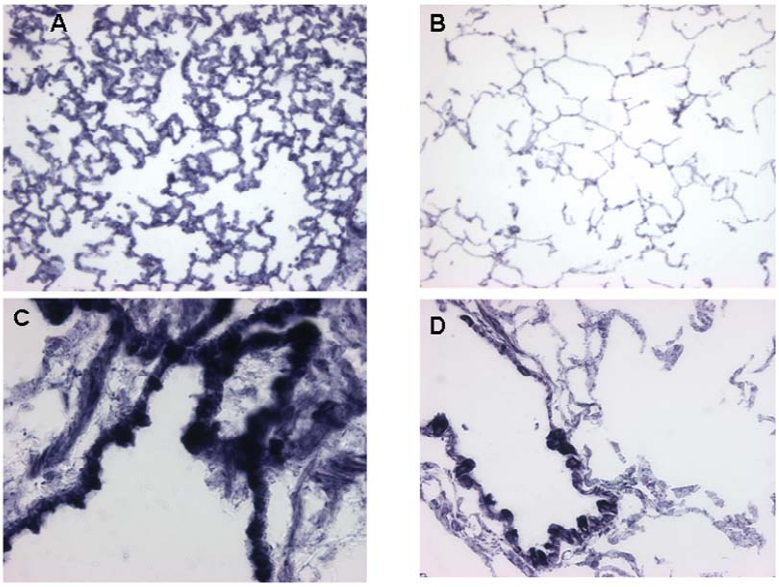

Figure 5 Influence of dopamine treatment on tissue viability. *Rats were treated with dopamine (A and C) or left untreated (B and D) as described in figure 1. The lungs were explanted and subjected to 8 hr of cold preservation. NADH staining of lung tissue was performed after 180 minutes of warm reperfusion. Note a more intensive NADH staining in dopamine treated lungs. The results of a representative experiment (n=5) is depicted. Original magnification was X10 in A and B and X40 in C and D.*

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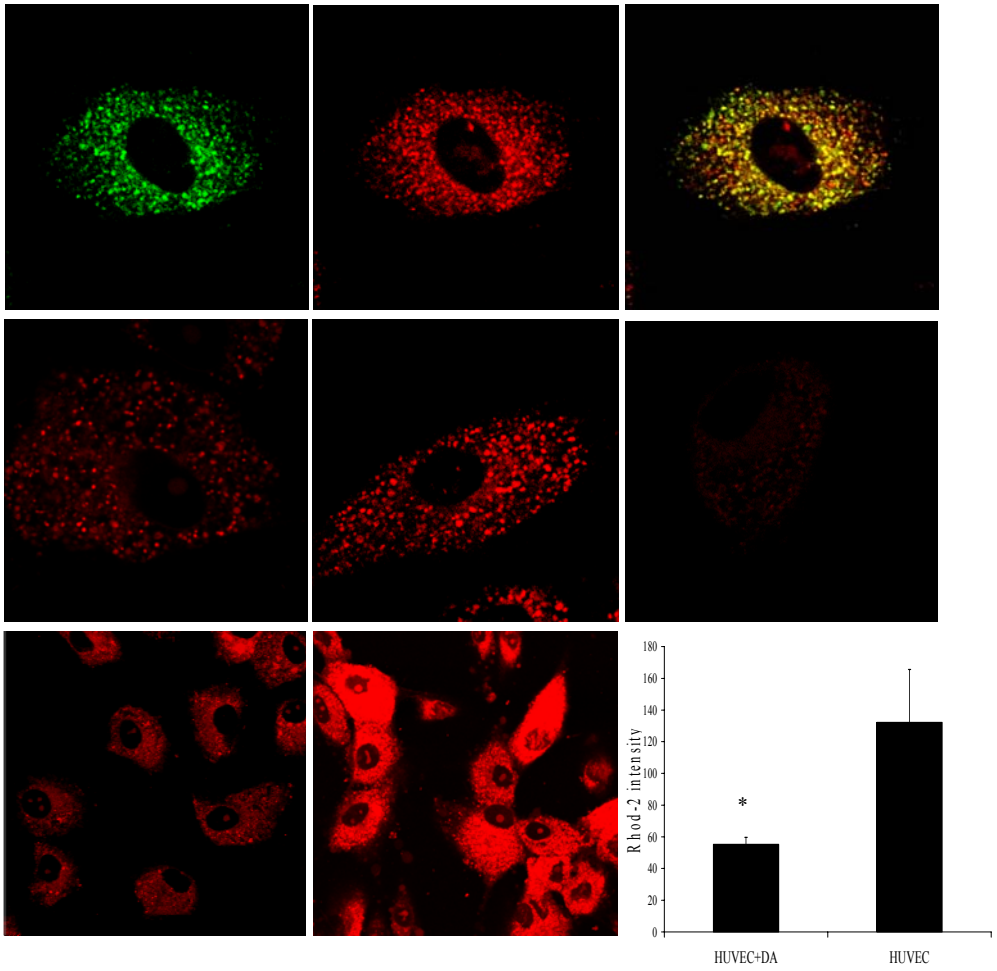

Figure 4 Ca^{2+} is accumulated in mitochondria during hypothermia. *HUVEC were incubated 2hrs prior to cold storage with 25µM DA or not. The cells were washed and labelled with Rhod-2 and Mitotracker-green directly before hypothermic preservation. 3hrs after initiation of hypothermia, the cells were analysed by confocal microscopy using the corresponding excitation and emission wave lengths. Specificity of Rhod-2 staining for mitochondrial Ca^{2+} was demonstrated by co-localization with Mitotracker-green and the absence of Rhod-2 staining when the cells were treated with CCCP to dissipate the mitochondrial membrane potential.*

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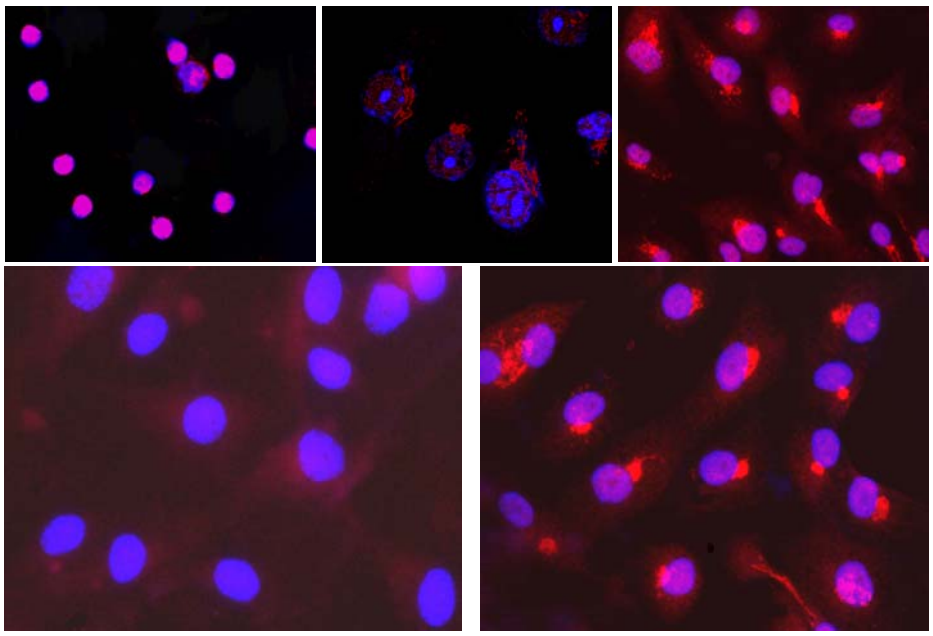

Figure 1 HMGB1 expression in endothelial cells*. Cultured HUVEC (upper two panels to the left) and freshly isolated PBMC (upper panel to the right) were stained for HMGB1 as described in the materials and methods section. Analysis of HMGB1 was performed by means of immune-fluorescence (upper panels to the left and to the right) and by confocal microscopy. Original magnification: 200x and 400x respectively. The lower panels show HMGB1 expression in untreated HUVEC (to the right) and dopamine treated HUVEC (to the left after 24 hrs of cold storage. Original magnification: 400x.*

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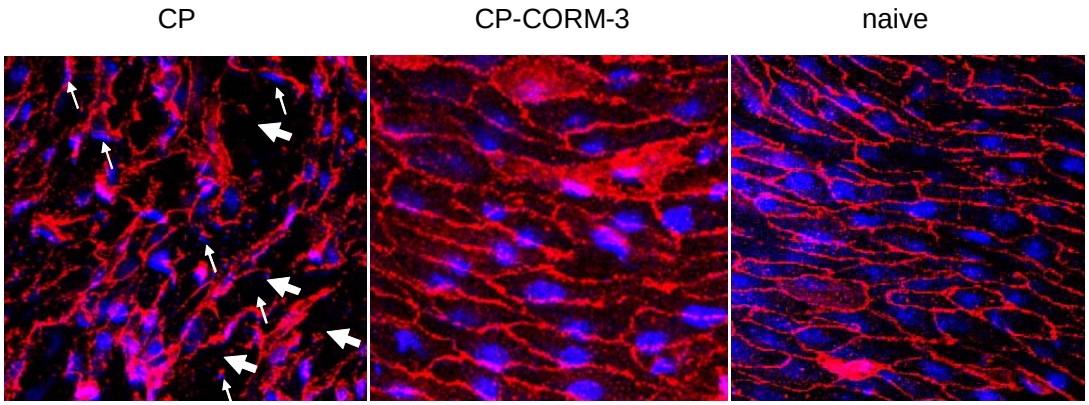

Figure 2 Influence of CORM-3 on endothelial integrity in aorta segments subjected to cold preservation. *Whole mount staining for CD31 (red) was performed as described in the Material and Method section. Nuclear staining is depicted in blue. Note that in the aorta segments subjected to cold preservation in the absence of CORM-3, endothelial cell morphology was irregular with intercellular gaps (bold arrows) appearing between adjacent endothelial cells. Also in the majority cells nuclear condensation and fragmentation (arrow) was observed (left panel). In contrast, aorta segments to which 50 µM of CORM-3 was added during cold preservation (middle panel) appeared to be similar to freshly isolated aorta segments not subjected to cold preservation (right panel). Original magnification: 200x*

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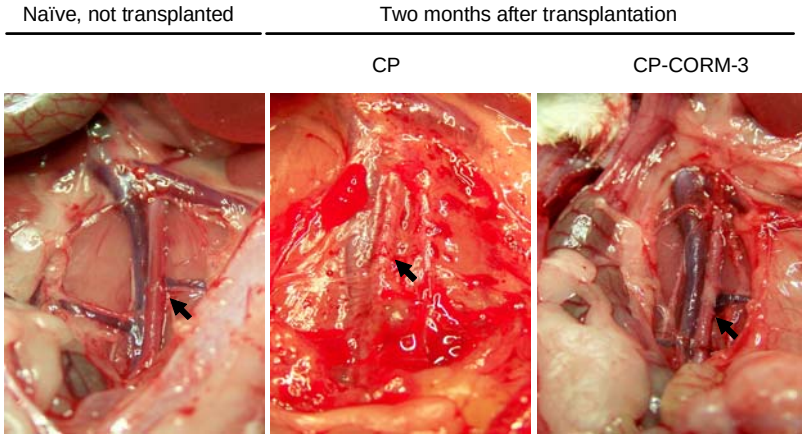
Figure 4 A



B

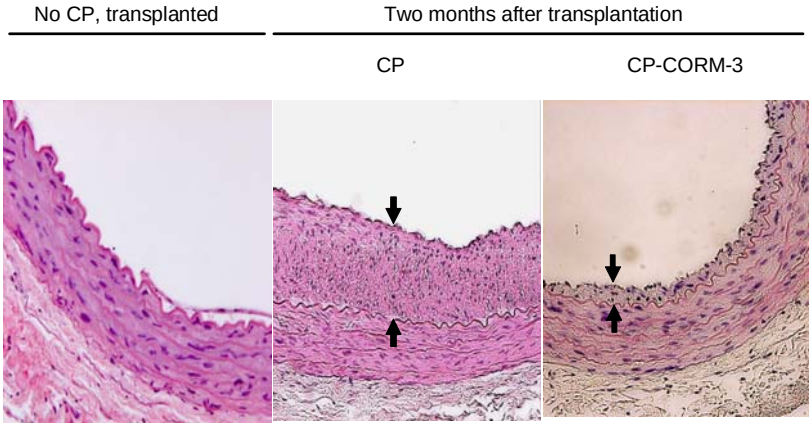


Figure 4 Addition of CORM-3 during cold preservation inhibits vascular remodeling. (A) *Syngeneic aorta transplantations were performed as described in the Materials and Methods section. The aortas were subjected to 24 hrs of cold preservation in the absence (CP) or presence (CP-CORM-3) of 50µM CORM-3. Two months after transplantation the grafts were harvested and macroscopically examined. Note the presence of fibrous tissue surrounding the graft (arrow) in the aortas subjected to cold preservation in the absence of CORM-3 (middle panel). This was not observed when 50 µM of CORM-3 was added during cold preservation (right panel). For comparison an aorta from a naïve non-transplanted rat is also depicted (left panel).* ***(B)*** *After macroscopic examination, the grafts were harvested and tissue sections were analysed by light microscopy using H&E staining. Representative photographs of transplanted grafts not subjected to cold preservation (No CP), subjected to cold preservation in the absence (CP) or presence of 50 µM of CORM-3 (CP-CORM-3) are depicted. Intimal hyperplasia is shown as the tissue in between the black arrows. Original magnification: 200x.*

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